A majority of the Food and Drug Administration’s Blood Products Advisory Committee (BPAC) voted to support a selective strategy to test blood donors for *Trypanosoma cruzi*, the agent of Chagas disease.

The Committee agreed that one negative test should allow future donations without further testing or risk-based questions to assess the potential for newly acquired infections. The Committee also determined that studies should continue to define the incidence of new infections in donors who previously screened negative. This approach was recommended by AABB (formerly known as the American Association of Blood Banks) and the American Red Cross.

A majority of the Committee also agreed with the FDA’s position that scientific data on the effectiveness of risk questions does not support a selective testing strategy in which donors who previously tested negative for *T. cruzi* antibodies are tested again only if their answers to these questions indicate the potential for new infection. Blood donations from individuals from endemic areas are the primary source of risk for *T. cruzi* infection from transfusion. Studies in the mid-1990s estimated that the rate of seropositive blood donors in the U.S. ranged from 1 in 5400 to 1 in 25,000, depending on where the studies were conducted. However, more recent studies suggest that these rates have increased in the areas where donor testing has been performed over a period of time. For example, a rate of 1 in 2000 was found recently in the Los Angeles metropolitan area. Transfusion transmission in endemic areas has been a major public health concern, and many countries considered endemic for *T. cruzi* infection screen blood donors for the presence of antibody.
Carlos Justiniano Ribeiro Chagas was a Brazilian physician. He discovered Chagas’ disease, also called *American trypanosomiasis* in 1909, while working at the Oswaldo Cruz Institute in Rio de Janeiro.

Chagas was the only researcher to describe completely the new infectious disease: its pathogen, vector (*Triatominae*), host, clinical manifestations and epidemiology. After graduating from medical school in 1902, he worked on ways to curtail malaria. He introduced an innovation, which consisted of using pyrethrum, an insecticide, to disinfect households. His published work on this method served as the basis of the global prevention of malaria.

In 1906, Chagas joined the Oswaldo Cruz Institute. While conducting field research, he observed the peculiar infestation of the houses with a large hematophagous insect of the genus *Triatoma*, a kind of “assassin bug” or “kissing” bug (barbeiro or “barber” in Portuguese), so called because it sucked the blood at night by biting the faces of its victims. He discovered that the intestines of these insects harbored a flagellate protozoa, a new species of the *Trypanosoma* genus. He was able to prove experi-
In the U.S. and Canada, only seven cases of transfusion-transmitted *T. cruzi* infections and five cases of infection from organ transplantation have been documented. However, transmission in immunocompetent patients is not likely to be apparent. In many cases, even if symptoms appear, infection may not be recognized. The FDA is aware that lookback studies conducted using the licensed ELISA test indicate that the risk of contracting *T. cruzi* by transfusion of a seropositive unit in the U.S. may be much lower than previously thought. The rate of transfusion transmission of *T. cruzi* in the U.S. continues to be uncertain because of the limited number of studies conducted to date. The rate of transfusion transmission remains under investigation.


**CHAGAS DISEASE AT A GLANCE**

- **16-18 million** people in the world are estimated to be infected with Chagas disease.
- **100 million** are at risk in 21 countries which includes about 25 percent of the population of Latin America.
- **50,000** deaths are due to Chagas disease each year.
- **1855** is the year when Chagas disease was first observed in the United States.
- **300,000** is the approximate number of immigrants to the United States infected with *T. cruzi*.
- **1:29,000** is the prevalence of *T. cruzi* antibody positive blood donations in the United States.

Source: WHO, CDC.

mentally that it could be transmitted to marmoset monkeys which were bitten by the infected bug. Chagas named this new parasite *Schizotrypanum cruzi*, in honor of Oswaldo Cruz (later renamed to *Trypanosoma cruzi*).

Chagas suspected that the parasite could cause human disease, due to the prevalence of the insect vector in human households and its habit of biting people. He took blood samples and discovered, for the first time the same *Trypanosoma* parasite in the blood of a three-year-old girl. He also observed parasitic inclusion in the brain and myocardium, which explained some of the clinical manifestations in diseased people, and he closed the proposed life cycle of the parasite by suggesting that the armadillo could be its natural reservoir. To complete his work on the pathology of the new disease, Chagas described 27 cases of the acute form of the disease and performed more than 100 autopsies on patients who exhibited the chronic form.
Severe traumatic brain injury (sTBI) case studies report high percentages of fatalities, as well as poor long-term prognoses in terms of cognitive abilities and patient quality of life. In particular, anemia has been shown to be injurious to the brain, and thus detrimental to patients with sTBI.

This study evaluated 30 patients who presented with sTBI to an adult critical care unit between January 2003 and July 2005. On average, patients received transfusion four days after injury. Patients were randomly assigned to three hemoglobin (Hb) threshold levels (8, 9, and 10 g/dL), and were transfused with 2 units of packed red blood cells when Hb levels dropped below those thresholds. Physiologic data and Hb levels were recorded after a stabilization period of one hour, and the change in brain tissue oxygen (PbtO2) was assessed as the primary outcome. Additionally, secondary outcomes included the dependence of baseline Hb concentration and PbtO2 on the relationship of transfusion and PbtO2, as well as the effect of transfusion on lactate pyruvate ratio (LPR) and brain pH as markers of cerebral metabolic state.

In terms of PbtO2, 57% of patients experienced a significant increase in PbtO2. However, no significant association was found between PbtO2 and baseline Hb. In addition, no significant association was found between a change in LPR and change in Hb levels, nor was an increase in brain pH observed. These results indicate that transfusion of packed red blood cells may improve brain tissue oxygenation, but does not have significant effects on cerebral metabolism.

Although the study showed a significant PbtO2 increase in 57% of patients, the authors noted that increases in PbtO2 were most prominent in patients with LPR greater than 25. Increased LPR has recently been found to be a marker of mitochondrial dysfunction, and therefore the improvements in PbtO2 may only be seen in patients with cerebral metabolic dysfunction. The study also concluded that the results may not be representative of whole brain metabolism, because the microdialysis monitors used in this study sampled only a small portion of the brain. Finally, the study sample was small, and the duration was too short to consider the long-term effects of prestored donor erythrocytes on oxygen delivery. Thus, more comprehensive studies will be needed to evaluate transfusion strategies in sTBI patients.

STUDY FINDS LACK OF EVIDENCE OF TRANSFUSION TRANSMISSION OF CREUTZFELDT-JAKOB DISEASE IN US

Since 2004, several reported transfusion transmissions of variant Creutzfeldt-Jakob disease (vCJD) in the United Kingdom have reawakened concerns about the possible risk of similar transmissions of nonvariant or classic forms of CJD.


The American Red Cross and other researchers conducted a study comprised of patients with a CJD diagnosis and a history of donating blood. Through a review of blood distribution and hospital records, the recipients of blood components from these donors were identified. The researchers then determined each recipient’s vital status and, if deceased, the cause(s) of death identified by matching the recipient’s personal identifiers with the Centers for Disease Control and Prevention’s National Death Index database. The researchers conducted such searches after recipients were enrolled in this study and annually thereafter for those who remained alive.

The study included a total of 36 blood donors who subsequently developed CJD and 436 recipients. Through 2006, 91 of these recipients were still alive, 329 were deceased, and 16 were lost to follow-up. After transfusion, these three groups had survived a total of 2096.0 person-years. A total of 144 recipients survived 5 years or longer after transfusion and 68 of them had received blood donated 60 or fewer months before the onset of CJD in the donor. The study identified no recipient with CJD.

The current results of this large, ongoing lookback study show no evidence of transfusion transmission of CJD, reinforcing the conclusion that the risk, if any, of transfusion transmission of prion disease by CJD donors is significantly lower than the comparable risk of such transmission by vCJD donors.

RED CROSS LEADERS LEAVE LEGACIES

YENSHEN HSUEH, MD: 1950-2009

Yenshen Hsueh, MD, the CEO of the American Red Cross River Valley Blood Services Region, died while vacationing in China during March 2009. Hsueh had managed the River Valley Blood Services Region for 13 years. The Region includes Louisville, mid-Kentucky, southern Indiana and southeastern Illinois, serving 56 hospitals.

Hsueh joined the American Red Cross Blood Services in 1985. He joined the Northern Ohio Blood Services Region as Medical Director in 1985 and moved to the Appalachian Region in Roanoke, Virginia in 1991 as Principal Officer and Chief Medical Officer. He assumed the Louisville-based CEO position in 1996.

Before joining the Red Cross, Hsueh developed a distinguished medical career. He was board certified in both anatomic and clinical pathology. He earned his medical degree from the Taipei University in Taiwan. Hsueh also studied medicine at the University of California. He finished his pathology training at Michigan State University with a Transfusion Medicine Fellowship at the University of Cincinnati.

In 2002, Hsueh was appointed Assistant Clinical Professor at the University of Louisville Medical School, Department of Laboratory & Clinical Medicine.

Hsueh is survived by his wife of 34 years, Mei, his son, John, and his daughter, Cindy.

A fund has been established in Dr. Hsueh’s name to carry on his legacy of service to patients, community and the American Red Cross. Donations will foster the development of an endowment to support a transfusion medicine fellowship with the American Red Cross.

Donations may be made to the American Red Cross Yenshen Hsueh, MD Medical Scholarship Fund, P.O. Box 1675, Louisville KY. 40201-1675.
Serologic screening for syphilis has been justified in part as a surrogate marker for infections caused by other pathogens such as human immunodeficiency virus (HIV). An American Red Cross Biomedical Services study assessed the current surrogate value of the test.

Researchers analyzed the testing results for American Red Cross blood donors between January 1, 2006, and December 31, 2007. All donations were tested according to standard procedures for markers of HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-lymphotropic virus (HTLV), syphilis, and other infections. The frequency of window-period (w-p) infections interdicted by syphilis testing was estimated.

The researchers found that there were significantly higher frequencies of HIV, HCV, hepatitis B surface antigen (HBsAg), and HTLV confirmed-positive donations among those with positive syphilis test results, although the sensitivity of syphilis test positivity in these groups was low. Among more than 3 million repeat donors with complete testing through reactive donation confirmation for both syphilis and HIV (anti-HIV and HIV RNA), 225 seroconverted for syphilis but not for anti-HIV or HIV RNA and 83 converted for HIV (anti-HIV or HIV RNA) but not for syphilis, with only 1 who converted for both syphilis and HIV, resulting in an incidence ratio of 150 (95% confidence interval, 21-1080) and a sensitivity of 1.2 percent. No syphilis seroconverters converted for HCV, HBsAg, or anti-HTLV.

The study concluded that syphilis testing presents no surrogate value for incident HCV, HBV, and HTLV infections and could only remove approximately 1 HIV w-p unit of every 148 million donations.


Treponema pallidum are Gram-negative, spirochete bacteria. Two spirochetes are shown on two sperm heads. Treponema is an obligate human parasite and is the causative agent of syphilis. It is a spirochete, a helical to sinusoidal, spiral-shaped, corkscrew-shaped, bacterium with outer and cytoplasmic membranes, a thin peptidoglycan layer, and periplasmic flagella. SEM X4000.

SYphilis Over 500 Years

1493-1495 - First cases of syphilis are reported in Europe.
1530 - Girolamo Frasastoro first derives the term "syphilis sive morbus gallicus" due to the rash associated with the disease.
1905 - Schaudinn identifies Spirocheta pallida as the causative agent of syphilis.
1911 - California becomes first state to require physicians to report syphilis cases. To assure confidentiality, reporting is done by number rather than name.
1932 - The United Public Health Service begins an infamous study of 412 impoverished African American men diagnosed with syphilis. They observed the course of the disease while withholding treatment.
1941 - Syphilis cases are first reported to the CDC.
Seven-day stored apheresis platelets (APs) were withdrawn from the US market after detection of two culture-positive units from 2571 tested at outdate in the PASSPORT surveillance study. The impact of this discontinuation on recipient safety was explored using mathematical modeling.

This study developed risk models for septic transfusion reactions (STRs) and transfusion-related acute lung injury (TRALI). Key assumptions were 400,000 annual APs transfused, equivalent STR risk for platelets (PLTs) stored for 5 days or more and zero for PLTs stored for less than 5 days, whole blood-derived PLTs (WBplts) administered in 5-unit pools, a 4.6-fold higher risk of false-negatives with surrogate versus culture-based bacterial testing, an AP TRALI risk between 1 per 1000 and 1 per 10,000, and a delay in TRALI risk reduction implementation in some centers by 6 to 12 months due to limited PLT availability.

The study found STR risk could increase, decrease, or remain the same depending on the percentage of inventory replaced by surrogate-tested WBplts versus culture-tested apheresis or whole blood PLTs. A delay in TRALI risk reduction implementation is likely to result in a comparable or greater risk during the delayed implementation period than the safety achieved with regard to STRs, even in the most favorable case scenario.

The study concluded that a comprehensive risk assessment should have been conducted before the decision to discontinue PASSPORT. Risk assessments using accepted methods (and actual data when available) should precede any major blood safety decisions.

On April 1-2, 2009, the Food and Drug Administration’s (FDA) Blood Products Advisory Committee (BPAC) met to advise the agency on blood donor screening and testing donors of human cells, tissues, and cellular- and tissue-based products for hepatitis B virus infection (HBV) by nucleic acid testing (NAT).

The FDA currently requires blood donors to be tested for HBV surface antigen, or HBsAg, and the antibody to HBV core antigen, anti-HBc. As of December 2008, three assays have approved indications for donor screening.

At the meeting, the American Red Cross presented data from an evaluation of the yield of HBV DNA-positive, seronegative donors using an automated HIV-1/HCV/HBV triplex assay. Data were collected on donations that were HBsAg and anti-HBc nonreactive using a combination of ID NAT and MP NAT of 16 donations. The study also focused on determining the rate and characteristics of HBV yield donors and the HBV screening claim approved by FDA for using MP NAT with a pool size of up to 16 donations.

The American Red Cross study detected nine HBV DNA-positive, HBsAg-negative and anti-HBc-negative donations using HBV NAT. The study produced comparable yields to other HBV yield studies performed in the U.S. Six of the nine donations were from immunized individuals (vaccine breakthrough cases) that had anti-HBs at index or shortly thereafter, and three of the nine were considered window-period donations. In addition, researchers noted that eight of the nine cases were detected using MP NAT with a pool size of 16. The study found that the data does not suggest a benefit of using a pool size smaller than 16, and the high yield of a MP of 16 has a value that compares to ultrasensitive HBsAg assays. The ninth case was only detected by ID NAT and not detected when using a smaller pool size of four or eight. Although ID NAT has additional value, currently it is not logistically feasible and is not supported by a yield perspective from the study, where ID NAT only identified one donor with a breakthrough infection.

Breakthrough infections often are characterized by low viral load and are asymptomatic. The rate of breakthrough infections among HBV-vaccinated individuals is unknown and may be detected with NAT window period modeling. The committee had an in-depth discussion questioning if the units from donors with apparent vaccine breakthrough are infectious. FDA officials noted that the estimated residual risk by modeling does not include vaccinated donors, breakthrough infections are unlikely to be detected without NAT and risk of transfusion-transmitted infections due to units from donors with breakthrough infections cannot be dismissed.

In a joint statement with the AABB, the American Red Cross stated that adopting HBV NAT will provide an "incremental improvement in HBV transfusion safety... from donors with acute infection whether previously vaccinated or naïve." The Red Cross and AABB explained that it is reasonable for FDA-licensed blood establishments to implement HBV MP NAT on a voluntary basis until the FDA mandates testing and that MP NAT with pool sizes of six or 16 should be used because there is no benefit to smaller MP sizes from either modeling studies or clinical studies. The organizations stated that they oppose a mandate for ID NAT at this time due to the relatively small increased yield observed in the Red Cross study, increased donor deferral and donation loss due to false positivity, and logistics of a six- to 16-fold increase in test volume and cost. A majority of the committee voted that breakthrough HBV infections should be presumed infectious pending further studies.

It was also noted that the absence of effective reimbursement mechanisms by which hospitals can recover the increased costs of blood safety initiatives, implemented voluntarily or after an FDA recommendation, remains a serious flaw in the regulatory process. HBV NAT is an example of such an initiative that will come as an unfunded mandate if FDA recommends its use.
HEPATITIS B
AT A GLANCE

2 billion people in the world have been infected with HBV.

400 million people in the world are chronically infected.

1 million people in the world die each year from HBV and related complications which equates to about 2 people per minute.

12 million Americans are infected with HBV.

1 million Americans are chronically infected.

5,000 Americans die from HBV and related complications each year.

Source: WHO, CDC, Hepatitis B Foundation.

M. DEAN ELFATH, MD: 1953-2009

Elfath was a medical graduate of Ain Shams University in Cairo and received a Masters in Business Administration from American Intercontinental University. After training in Pathology and Transfusion Medicine at the University of Massachusetts, he served as Associate Medical Director of the Blood Bank and Hematology Laboratory, University of Maryland. Elfath was the principal investigator of many studies designed to improve blood technologies, resulting in more than 100 abstracts and publications. He was a member of AABB (formerly the American Association of Blood Banks), the American Board of Pathology, International Transfusion Consultants, the American Society for Apheresis, the American Society for Hematology, and the International Society of Blood Transfusion. He was a fellow for the American Society of Clinical Pathologists and the College of American Pathologists, as well as a frequent presenter at many national and international meetings.

At the American Red Cross, Elfath brought renewed interest to the research program at the Mid-Atlantic Region and was innovative in his approaches to blood collection and storage. Working closely with manufacturers in collaborative research and development projects, Elfath brought great energy and an encouraging attitude to all the Region staff and to those whose lives he touched.

Having lost his father in the 1967 Six-Day War, Elfath remembered childhood experiences when the Red Cross and Red Crescent provided humanitarian services to the many refugees in the area. He spoke passionately about how the American Red Cross was an instrument for good in the world and was proud of his time with the organization.

He died in his home in Cairo, Egypt.
Individual patient data-based meta-analysis was conducted with a pooled data set provided through six published and one unpublished cohorts. Outcomes in recipients of peripheral blood or bone marrow transplantation for hematologic malignancies were evaluated. A multivariate Cox model was used to adjust differences in outcomes of patients receiving ABO-matched grafts with those receiving major, minor, or bidirectional mismatched grafts. Considering multiple testing, p values of less than 0.05 and 0.001 were considered significant for the primary and secondary endpoints, respectively.

In all, 1208 cases, including 697 ABO-matched and 202 major, 228 minor, and 81 bidirectional mismatched transplants, were analyzed. Overall, the adverse impact of ABO matching on overall survival (OS), as a primary endpoint, was not observed (adjusted hazard ratios [95% confidence intervals]: major, 1.03 [0.82-1.30], p = 0.81; minor, 1.19 [0.97-1.47], p = 0.10; bidirectional, 1.25 [0.91-1.72], p = 0.17). Among related stem cell recipients, ABO matching had no significant influence on OS, while the minor and bidirectional mismatched groups among unrelated stem cell recipients exhibited lower OS with marginal significance, especially in patients with acute leukemia, patients who received transplants after 1998, and patients who underwent transplants at Asian centers.

This meta-analysis demonstrates no adverse association between any ABO mismatching and survival. However, marginally lower OS was found in recipients of minor or bidirectional mismatched grafts from unrelated donors suggested the need for larger studies focusing on unrelated transplants.

The study was conducted by researchers from Kyoto University (Japan), Aichi Cancer Center Research Institute (Japan), Nagoya University (Japan), American Red Cross Biomedical Services, Temple University, Blood Transfusion Centre of Slovenia, University of Rochester Medical Center-Rochester, Northwestern University, and Kyungpook National University Hospital (Korea).

A new study contends that preoperative patients’ characteristics can predict the need for perioperative blood component transfusion in cardiac surgical operations. The aim of this prospective observational study was to identify perioperative patient characteristics predicting the need for allogeneic packed red blood cell (PRBC) transfusion in isolated primary coronary artery bypass grafting (CABG) operations.

105 patients (97 males and 8 females) undergoing isolated, first-time CABG were reviewed for their preoperative variables and followed for intraoperative and postoperative data. The mean age was 58.28 +/- 10.97 years. Regression logistic analysis was used for identifying the strongest perioperative predictors of PRBC transfusion.

PRBC transfusion was used in 71 patients (67.6%); 35 patients (33.3%) needed > 2 units and 14 (13.3%) of these needed > 4 units.

Univariate analysis identified female gender, age > 65 years, body weight <70 Kg, BSA <1.75 m2, BMI <25, preoperative hemoglobin <13 gm/dL, preoperative hematocrit <40%, serum creatinine > 100 mmol/L, Euro SCORE (standard / logistic) > 2, use of CPB, radial artery use, higher number of distal anastomoses, and postoperative chest tube drainage > 1000 mL as significant predictors. The strongest predictors using multivariate analysis were cardiopulmonary bypass (CPB) use, hematocrit, body weight, and serum creatinine.

The study found that the predictors of PRBC transfusion after primary isolated CABG are use of CPB, hematocrit <40%, weight <70 Kg, and serum creatinine > 100 mmol/L. The authors state that further study of such predictors could lead to better utilization of blood bank resources and cost-efficient targeted use of blood conservation modalities.

THE JAL ANTIGEN (RH48) IS THE RESULT OF A CHANGE IN RHCE THAT ENCODES Arg114Trp

The JAL antigen (Rh48) was discovered more than 30 years ago when it caused hemolytic disease of the fetus and newborn in an African American family. A decade later it was found to cause hemolytic disease of the fetus and newborn in a Caucasian family. The presence of the same low-prevalence antigen in two different ethnic groups is rare, but additional JAL+ people in both groups were subsequently identified. This study was undertaken to investigate the RH gene(s) responsible for expression of JAL and to determine the structural relationship between JAL and other Rh antigens.

Samples from 17 JAL+ people were included: 2 Caucasian, 6 African American, 7 African Brazilian, 1 Caribbean, and 1 Puerto Rican. RHCE and RHD were investigated at the genomic level, and Rh cDNAs were cloned and sequenced for some samples.

Caucasian JAL+ probands had RHCE*Ce, while JAL+ probands with African ancestry had RHCE*ce, but all had a nucleotide 340C>T change in Exon 3 of RHCE predicted to encode Arg114Trp. The JAL-encoding RHCE*ce also had 733C>G (Leu245Val) and was linked to conventional RHD or to RHD*DAUo.

The researchers concluded that JAL+ results from a nucleotide 340C>T (Arg114Trp) on either a Ce or ce background. Homology modeling of the JAL+ RhCE protein suggests that the Arg114Trp change eliminates a critical loop-stabilizing H-bond between the side chain of Arg114 and the e-specific amino acid Ala226. Additionally, accommodation of the bulky tryptophan would disrupt the conformation of the extracellular loops containing C/c- and e-specific amino acids, providing a structural hypothesis for the simultaneous altered expression of C/c, e, and V/VS antigens.

Positive Direct and Indirect Antiglobulin Tests Associated with Oxaliplatin Can Be Due to Drug Antibody and/or Drug-Induced Nonimmunologic Protein Adsorption

Two patients were suspected of having immune hemolytic anemia (IHA) due to oxaliplatin. A related drug, cisplatin, is known to cause nonimmunologic protein adsorption (NIPA). Studies were performed to determine the presence of oxaliplatin-dependent antibodies in addition to oxaliplatin-induced NIPA.

Sera and eluates from the two patients were tested against red blood cells (RBCs) treated with oxaliplatin, cisplatin, and carboplatin (another platinum drug). Sera were also tested against untreated RBCs in the presence of the same drugs. Testing with pooled normal sera and anti-human albumin was used to demonstrate the presence of NIPA. Oxaliplatin-treated RBCs sensitized with the patients’ sera and pooled normal sera were tested by a monocyte monolayer assay (MMA) to determine potential clinical significance.

Both patients had high-titer antibodies to oxaliplatin in their sera that reacted with oxaliplatin-treated RBCs and with untreated RBCs in the presence of oxaliplatin. RBCs treated with oxaliplatin, cisplatin, and carboplatin all demonstrated NIPA (pooled normal sera and anti-human albumin were reactive to low titers). NIPA was also detected in tests with untreated RBCs in the presence of oxaliplatin and cisplatin. Lower-titer reactivity of both patients’ sera with cisplatin may have been due to NIPA and/or cross-reactivity of anti-oxaliplatin with cisplatin. MMAs were weakly positive due to NIPA and more strongly positive due to oxaliplatin antibodies.

Two patients with IHA were demonstrated to have oxaliplatin-dependent antibodies. Oxaliplatin was also shown to cause NIPA. The drug-dependent antibody and/or the drug-induced NIPA could have contributed to the patients’ hemolytic anemia.

Westhoff CM, Vege S, Wylie D, Nickle P, Lomas-Francis C, Hue-Roye K, and Reid ME. The JAL antigen (Rh48) is the result of a change in RHCE that encodes Arg114Trp. Transfusion. 2009. 49:725-732.