A. Immunemodulation

How to make an auto-nosode from stool, saliva or urine

Auto-Nosode Preparation

Materials needed:
Sterile urine containers
10mL sterile glass empty vials
10mL syringes
18G x 1” needle
Sterile water
0.3cc insulin syringes
Filter needle 0.2 micron

Fill sterile empty vials with 10mL of water and label each as C1, C2, C3, etc up to C12.

Fill 2 sterile urine cups with 10mL with sterile water.

Mix stool with tap water in a sterile urine container. Allow to incubate at room temperature for 48 hours. After 48 hours, mix thoroughly and draw up 1mL into a syringe.
Put the 1mL mixture into a new sterile urine container with 10mL sterile water. Mix thoroughly.

With a new syringe and filter needle, draw up 1mL of mixture. Take the filter needle off and squirt into a new sterile urine cup with 10cc sterile water. Mix thoroughly.

Draw up 0.01mL with the 0.3mL insulin syringe and add to sterile vial labeled C1 (pre-filled with 10mL sterile water). Succuss (hit bottom of bottle against palm of hand) 50 times.

Draw up 0.01mL from C1 and add to vial C2, succuss 50 times.

Draw up 0.01mL from C2 and inject into C3 and succuss 50 times.

Continue diluting until C12.

B. Important Reading:

Lyme Treatment Guidelines by Dr Burrascano - Lyme disease
Artesunate source:
Asia:
Tri-Health
This is to reply your request of Artesunate for injection.

PRODUCT: Artesunate for Injection
MANUFACTURER: Guilin Pharmaceutical Co. Ltd.
DOSAGE FORM: 60mg/vial
PRICE: $12.00 per vial
SHIPMENT: $40.00 via Express Mail Service for each shipment from China

HOW TO ORDER: Please place your order via email address
at TriHealth2000@Yahoo.com.HK with following information,
(1) Product name and quantity;
(2) Recipient (consignee) name, address, and phone number;
(3) Your payment.

PAYMENT: We accept your credit card (Visa or MasterCard) for your payment.
Following credit card information is requested for the payment, (1) Credit card
number; (2) Expiration Date; (3) Name of the card holder; (4) Cardholder's credit
card statement address if it is different from your shipping address; (5) Security
code in your credit card. It is a 3-digit number following to your credit card
number in the signature strip on the back of your credit card.

SHIPMENT: Your order will be shipped to you via Express Mail Service (EMS)
soon after your order is confirmed.

Please let me know if I can be of any further assistance!

Sincerely,
James Eksu, MD.
International Sales and Marketing
Tri-Health Co. Ltd.
A Subsidiary of Canton Pharmaceuticals, Inc.
Hong Kong
Email: TriHealthHK@Yahoo.com.HK

C. Other herbal remedies that are useful (observation
by Scot Forsgren)
Scott@BetterHealthGuy.com

NutraMedix -
https://www.nutramedix.com/

Burbur - excellent for detoxification; can be used every 10 minutes to help pull someone out of a Herxheimer reaction
Samento / Banderol - this is a good antimicrobial combination and was studied by Eva Sapi's team with positive results. Note have to be separated by 15-20 minutes.
Cumanda - another broad spectrum antimicrobial
Enula - seems to be helpful for Babesia and protozoa
Houttuynia - seems to be helpful for Bartonella
Stevia - this is the stevia used in Eva Sapi's research on Stevia for Lyme

Beyond Balance -
http://www.beyondbalanceinc.com/

BB-1, BAR-1, and BAB-2 - these may be helpful for the "Big 3"
TOX-EASE GL and TOX-EASE - detoxification support
BFM-1 - biofilm (generally later in treatment)
CYFLACALM II - I really like this one for nervous system and brain related inflammation

There are others in this line I really like too like MYCOREGEN for fungal issues, MC-CH for CPn, ENL-MC for Mycoplasmas, etc.

Byron White Formulas -
http://www.byronwhiteformulas.com/

A-L Complex, A-BART, A-BAB - these may be helpful for "the Big 3"
A-FNG - may be helpful for fungal issues
A-P - may be helpful for parasites
A-MYCO - may be helpful for Mycoplasmas
A-CPN - may be helpful for Chlamydias

**Supreme Nutrition** -

- Takesumi Supreme - my favorite binder
- Morinda Supreme (noni) - a good broad spectrum antimicrobial
- Melia Supreme (neem) - a good broad spectrum antimicrobial
- Gloden Thread Supreme (coptis) - a good broad spectrum antimicrobial

I use these antimicrobials generally when people are through the more specific, targeted treatments (Beyond Balance, etc.) and need something broad-spectrum to maintain an antimicrobial focus.

**Maypa Herbals** -

- Formula L or L Plus - Borrelia
- Formula Bart
- Formula Bab Plus

I find the Maypa remedies are very well tolerated and people find they work well.

Other things I like:

- Biobotanical Research Biocidin, Olivirex, and GI Detox (good binder)
- ACZ Nano spray - liquid zeolite that tests very well for many (Daniela is sending me the new zeolite you like to test on people). Another one I have been using is CytoDetox but I still think I like the ACZ Nano better so far.
- RESTORE - seems to be excellent for GI/leaky gut support. [restore4life.com](http://restore4life.com)

Devices I like:

- MAS PEMF
C. Astragalus


**D. AstraSmile**


**Herbs with Anti-Lyme Potential**

E. Coriandolo

Cilantro for detox of lead and aluminum

1. Y. Omura et al: Preventative Effects of Chinese Parsley on Aluminum Deposits in ICR Mice
   Acupuncture & Electro-Therapeutics Research 28 (1/2) 1-44 (2003)


The preventive effect of Chinese parsley on aluminum (Al) deposition was investigated in male ICR mice exposed to Al. Seven weeks old ICR male mice were exposed to 1000 ppm Al as Al chloride in drinking water for 39 days. Administration of Chinese parsley to mice by gastric intubation was performed for 25 days from 14 days after beginning of Al exposure to the end of experiment. After 39 days, the mice were sacrificed for the comparison of Al distribution. The localized Al in various tissues was analyzed by kinetic differentiation mode of HPLC.

After Al exposure, Al was found to accumulate in the brain, kidney and femur. Localized Al deposition in brain was significantly decreased by the administration of 2.4mg/body of Chinese parsley as shown in Fig.1. The similar results were obtained in the femur (Fig.2). Surprisingly, Al levels in femur on Chinese parsley administered group were lower than that on control. Orally administered Chinese parsley is effective at reducing the deposition of Al in the tissues. These findings suggest the possibility that Chinese parsley may be useful as a natural antidote for Al intoxication.

Fig.1 Effect of Chinese parsley on Al concentration in the brain
Fig.2 Effect of Chinese parsley on Al concentration in the femur

Aga M; Iwaki K; Ueda Y; Ushio S; Masaki N; Fukuda S; Kimoto T; Ikeda M; Kurimoto M

The preventive effect of Coriandrum sativum, Fam. UMBELLIFERAE (Chinese parsley) on lead deposition was investigated in male ICR mice given lead (1000 ppm) as lead acetate trihydrate in drinking water for 32 days. Administration of Chinese parsley to mice by gastric intubation was performed for 25 days from day 7 after the start of lead exposure up to the end of the experiment. The mice were then sacrificed for comparison of lead distribution. The lead reached its highest concentration in the femur but localized lead deposition in the femur was significantly decreased by meso-2,3-dimercaptosuccinic acid (DMSA), a chelating agent used as a positive control to validate this experimental model. Administration of Chinese parsley also significantly decreased lead deposition in the femur and severe lead-induced injury in the kidneys. In addition, urinary excretion of delta-aminolevulinic acid (ALA) which is known to increase with lead intake was significantly decreased after administration of Chinese parsley. The MeOH extract of Chinese parsley also reduced lead-induced inhibition of delta-aminolevulinic acid dehydratase (ALAD) activity in vitro. These results suggest that Chinese parsley has suppressive activity on lead deposition, probably resulting from the chelation of lead by some substances contained in Chinese parsley.


Removal and preconcentration of inorganic and methyl mercury from aqueous media using a sorbent prepared from the plant Coriandrum sativum (National Center for Compositional Characterization of Materials (CCCM), Bhabha
4. Antimicrobial activity of individual and mixed fractions of dill, *cilantro*, coriander and eucalyptus essential oils  

Delaquis PJ; Stanich K; Girard B; Mazza G

Essential oils from dill (Anethum graveolens L.), coriander (seeds of Coriandrum sativum L.), *cilantro* (leaves of immature C. sativum L.) and eucalyptus (Eucalyptus dives) were separated into heterogeneous mixtures of components by fractional distillation and were analyzed by gas chromatography-mass spectroscopy. Minimum inhibitory concentrations against gram-positive bacteria, gram-negative bacteria and Saccharomyces cerevisiae were determined for the crude oils and their fractions. Essential oil of *cilantro* was particularly effective against Listeria monocytogenes, likely due to the presence of long chain (C6-C10) alcohols and aldehydes. The strength and spectrum of inhibition for the fractions often exceeded those determined in the crude oils. Mixing of fractions resulted in additive, synergistic or antagonistic effects against individual test microorganisms.

5. Role of mercury (Hg) in resistant infections & effective treatment of Chlamydia trachomatis and Herpes family viral infections (and potential treatment for cancer) by removing localized Hg deposits with Chinese parsley and delivering effective antibiotics using various drug uptake enhancement methods. 


Omura Y, Beckman SL.  Heart Disease Research Foundation, New York, USA.

Abstract
The authors found that antibiotics used to treat various infections often were in effective in the presence of abnormal localized deposits of heavy metals like Hg and Pb, which were often observed to co-exist with Chlamydia trachomatis, Herpes Simplex Types I & II, Cytomegalovirus (CMV), and other microorganisms. Our earlier research revealed that despite rigorous treatment with antibiotics together with various drug uptake enhancement
nt techniques, subjects who had been treated for Chlamydia trachomatis infections, seemingly successfully with disappearance of their symptoms, were often experiencing recurrences within several months after completion of their treatment despite taking precautions against reinfection. Careful examination of the entire body of these symptomfree patients with the Bi-

Digital ORing Test revealed that the Chlamydia trachomatis had retreated to 3 approximately 5 hiding places with localized increase in uric acid levels: 1) sublingual caruncle, 2) a small round area in the right and/or left axillae, 3) the genitals (Corona Glandis area of the Glans Penis at the Fossa Navicularis of the urethra in the male, and near the orifice of the urethra in the female), 4) Insulinlike Growth Factor positive horizontal lines, particularly above and below the knees, 5) the maxillary, ethmoid and frontal sinuses and the horizontal lines at the base of the nostrils (particularly small areas where Insulin-like Growth Factors exist). We found that all these areas contain Insulinlike Growth Factors I & II which are reduced in the presence of infection. Even when drug uptake of antibiotics was selectively increased in these 3 approximately 5 areas by various drug uptake enhancement methods developed by the 1st author, still the infection persisted. In the spring of 1995, use of Chinese parsley for successful elimination of Hg deposits existing in various organs of the first author as the result of the decay of radioactive Thallium 201 injected for cardiac SPECT, was accidentally discovered after eating Vietnamese soup, which happened to contain Chinese parsley, also called cilantro. We also found Chinese parsley accelerates the excretion of Hg, Pb, and A1 from the body through the urine. Our subjects were given a course of antibiotics (Doxycycline for Chlamydia trachomatis infection) or antiviral agents (EPA with DHA for Herpes Family Viruses) together with Chinese parsley. Since these vegetable/herbs were eaten, the amount of effective substance absorbed varied and some people did not like the taste of these relatively large amounts of either cooked or raw parsley or its juice, but together with effective antibiotics delivered by drug uptake enhancement methods to the infected areas, the substances worked synergistically, rapidly reducing the generalized symptoms and infection. The microorganisms retreated to the 3 approximately 5 areas listed above where, with continued treatment, they were significantly reduced, but not completely eliminated. Because of these problems, a pharmaceutical company was asked to produce a Chinese parsley tablet containing a controlled amount in a highly absorbable form. When 11 subjects were treated with Doxycycline for Chlamydia trachomatis infection, or antiviral agents (EPA with DHA) for Herpes Family Viruses, drug uptake enhancement methods to selectively increase delivery of the drugs to the affected areas, and Chinese parsley tablets to remove
the heavy metal deposits, the last traces of the infections and clinical symptoms disappeared completely. Therefore we hypothesized that the infectious microorganisms mentioned above, somehow utilize the Hg or Pb to protect themselves from what would otherwise be effective antibiotics, and/or that heavy metal deposits in some way make antibiotics ineffective. Since the microorganisms retreat to areas in which Insulinlike Growth Factors I & II normally exist, they may be utilizing them for their own growth and multiplication.

PMID: 8686573 [PubMed indexed for MEDLINE]

6. Significant mercury deposits in internal organs following the removal of dental amalgam, & development of pre-cancer on the gingiva and the sides of the tongue and their represented organs as a result of inadvertent exposure to strong curing light (used to solidify synthetic dental filling material) & effective treatment: a clinical case report, along with organ representation areas for each tooth. Acupunct Electrother Res. 1996 Apr-Jun;21(2):13360.

Omura Y, Shimotsuura Y, Fukuoka A, Fukuoka H, Nomoto T.

Heart Disease Research Foundation, New York, USA.

Abstract
Because of the reduced effectiveness of antibiotics against bacteria (e.g. Chlamydia trachomatis, alphaStreptococcus, Borrelia burgdorferi, etc.) and viruses (e.g. Herpes Family Viruses) in the presence of mercury, as well as the fact that the 1st author has found that mercury exists in cancer and precancer cell nuclei, the presence of dental amalgam (which contains about 50% mercury) in the human mouth is considered to be a potential hazard for the individual's health. In order to solve this problem, 3 amalgam fillings were removed from the teeth of the subject of this case study. In order to fill the newly created empty spaces in the teeth where the amalgams had formerly existed, a synthetic dental-filling substance was introduced and to solidify the synthetic substance, curing light (wavelength range reportedly between 400 and 520 nm) was radiated onto the substance in order to accelerate the solidifying process by photopolymerization. In spite of considerable care not to inhale mercury vapor or swallow minute particles of dental amalgam during the process of removing it by drilling, mercury entered the body of the subject. Prec
autions such as the use of a rubber dam and strong air suction, as well as frequent water suctioning and washing of the mouth were insufficient. Significant deposits of mercury, previously nonexistent, were found in the lungs, kidneys, endocrine organs, liver, and heart with abnormal lowvoltage ECGs (similar to those recorded 13 weeks after i.v. injection of radioisotope Thallium-201 for Cardiac SPECT) in all the limb leads and V1 (but almost normal ECGs in the precordial leads V2 to V6) the day after the procedures were performed. Enhanced mercury evaporation by increased temperature and microscopic amalgam particles created by drilling may have contributed to mercury entering the lungs and G.I. system and then the blood circulation, creating abnormal deposits of mercury in the organs named above. Such mercury contamination may then contribute to intractable infections or precancer. However, these mercury deposits, which commonly occur in such cases, were successfully eliminated by the oral intake of 100 mg tablet of Chinese parsley (Cilantro) 4 times a day (for average weight adults) with a number of drug-uptake enhancement methods developed by the 1st author, including different stimulation methods on the accurate organ representation areas of the hands (which have been mapped using the BiDigital ORing Test), without injections of chelating agents. Ingestion of Chinese parsley, accompanied by drug-uptake enhancement methods, was initiated before the amalgam removal procedure and continued for about 2 to 3 weeks afterwards, and ECGs became almost normal. During the use of strong bluish curing light to create a photo-polymerization reaction to solidify the synthetic filling material, the adjacent gingiva and the side of the tongue were inadvertently exposed. This exposure to the strong bluish light was found to produce precancerous conditions in the gingiva, the exposed areas of the tongue, as well as in the corresponding organs represented on those areas of the tongue, and abnormally increased enzyme levels in the liver. These abnormalities were also successfully reversed by the oral intake of a mixture of EPA with DHA and Chinese parsley, augmented by one of the noninvasive drug-uptake enhancement methods previously described by the 1st author, repeated 4 times each day for 2 weeks.

PMID: 8914687 [PubMed indexed for MEDLINE]

6. “Cilantro—Culinary Herb or Miracle Medicinal Plant”
F. Chlorella

BioPure uses a patented process by which stiff structural elements of the cell wall are cracked using sound waves. This keeps the extra cellular chlorella barrier intact, thus protecting all of the cell’s organelles. It has a better absorption of toxins, but is harder to digest than some other forms of Chlorella. Super nutrient: with 50-60% amino acid content. Methylcobolamin - the most easily absorbed and utilized form of B12, B6, minerals, chlorophyll, beta-carotene etc.

Directions for use: 4 Tablets 2 to 3 times per day with meals, or as directed by your practitioner. For mold/biotoxin binding/elimination and active phases of mercury/lead detox, use higher dosages ( 20-30 tbl 3-4 times per day, typically 30 min before meals or at bedtime)

Both C.pyrenoidosa (better absorption of toxins, but harder to digest) and C.vulgaris (higher CGF content – see below, easier to digest, less metal absorbing capability) are available. A scientific literature list is available on www.KlinghardtAcademy.com. Be aware that there are huge differences in quality. We only recommend BioPure chlorella.

Chlorella has multiple published health inducing effects:

- **Toxin binding** (mucopolysaccharide membrane) all known toxic metals, environmental toxins such as dioxin and others
- **Antiviral** (especially effective against the cytomegaly virus from the herpes family)
- Repairs and activates the body’s detoxification functions:
  
  - Dramatically increases intra-cellular reduced glutathion,
  - Sporopollein is as effective as cholestyramin in binding neurotoxins and more effective in binding toxic metals then any other natural substance found.
• Various peptides restore coeuruloplasmin and metallothioneine,
• Lipids (12.4 %) alpha-and gamma-linoleic acid help to balance the increased intake of fish oil during our detox program and are necessary for a multitude of functions, including formation of there peroxisomes.
• Methyl-coblolanine is food for the nervous system, restores damaged neurons and has its own detoxifying effect.
• Chlorella growth factor helps the body detoxify itself in a yet not understood profound way. It appears that over millions of years chlorella has developed specific detoxifying proteins and peptides for every existing toxic metal.
• The porphyrins in chlorophyll have their own strong metal binding effect. Chlorophyll also activates the PPAR-receptor on the nucleus of the cell which is responsible for the transcription of DNA and coding the formation of the peroxisomes (see fish oil), opening of the cell wall (unknown mechanism) which is necessary for all detox procedures, normalizes insulin resistance and much more. Medical drugs that activate the PPAR receptor (such as pioglitazone) have been effective in the treatment of breast and prostate cancer.
• **Super nutrient**: 50-60% amino acid content, ideal nutrient for vegetarians, methylcobolamin - the most easily absorbed and utilized form of B12, B6, minerals, chlorophyll, beta carotene etc.
• **Immune system strengthening**
• **Restores bowel flora**
• **Digestive aid (bulking agent)**
• **Alkalining agent (important for patients with malignancies)**

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**Literature on Heavy metal detoxification and Chlorella**

*(selected by Dietrich Klinghardt MD, PhD till 2008)*

We only use and recommend the pristinely grown sound cracked chlorella from BioPure. There has been trouble with other products.

**Chlorella safety**

500 Gramm Chlorella per day in experiment without serious side effects except bloatedness (Algae Feeding in Humans  R.Powell et al, J of Nutrition 75: 61, pg 7-12). Exempt in Japan from necessity of further safety studies

NIN report: no LD 50 in rats

South Korea: 4000 tons of chlorella used annually by humans without reports of worrisome side effects

Over 10 Million people take chlorella as daily supplement (ref 335) – Mason R: Altern Compl Therap, 2001;7 (3):161-165

**Chlorella and metal detox**

**Uranium**


**Lead**

Cadmium


Mercury


Klinghardt, D.: Algenpraeparat hilfreich bei der Amalgamausleitung Erfahrungsheilkunde Band 48, Heft 7, Juli 1999


Parachlorella beyerinckii CK-5 is found to accelerate excretion of methyl-mercury both into feces and urine: “Japan Society for Bioscience, Biotechnology and Agro-chemistry” (JSBBA: http://www.jsbba.or.jp) Meeting in Nagoya City, Japan, March 29~30, 2008.

Krisenon J: Inaugural dissertation (=PhD thesis) Fachbereich Chemie Universitäet GH Essen, 2002 (pp. 230, 231, 232, 236, 247, Ref. 922, 923, 924 u. 926, 1000-1003): Chlorella and CVE remove mercury, tin, antimonum, bismuth and arsenic (toxins) from the bloodstream and from the oral cavity

**Chlorella used traditionally to save lives after environmental catastrophes**


**CVE: treatment of intestinal infections (Listeria, pathogenic e.coli and CMV) and lead toxicity:**


**Chlorella in cancer therapy**


**Chlorella and chemical detox**

Effect of chlorella pyrenoidosa on fecal excretion and liver accumulation of polychlorinated dibenzo-p-dioxin in mice. Chemosphere 2005;59 297-304


Morita, K., Matsueda T., Iida, T., Hasegawa, T.: Chlorella accelerates *dioxin* excretion in rats. *Journal of nutrition* 129 (9): 1731-6, 1999


**Chlorella as general supplement**

*Over 10 Million people take chlorella as daily supplement* (ref 335) – Mason R: Altern Compl Therap, 2001;7 (3):161-165


CGF - optimal facial development, optimal skeletal growth and development of intelligence:


R.Pratt et al :Production of thiamine, riboflavin, folic acid and biotin by chlorella vulgaris und chlorella pyreneidosa  J of Pharmaceutical Sciences Vol 54, No.6, June 1965: chlorella contains significant amounts of: Vit B2, B3, methyl B12, D-3, Vit K, Vit C, Vit E, beta carotine and other carotinoids, all essentiell aminoacids, magnesium, iron, potassium, chlorophyll

Tokuyasu, M.: Examples of diets for infant’s and children’s nutritional guidance, and their effects of adding chlorella and C.G.F. to food schedule. *Totori City, Japan*: Comference proceedings at the


**The chlorella membrane: contents and properties**


**Pregnancy and Breastfeeding: the protective effect of chlorella**

S.Nakano et al: Maternal-fetal distribution and transfer of dioxins in pregnant women in Japan, and attempts to reduce maternal transfer with Chlorella (Chlorella pyrenoidosa) supplements Chemosphere, April 2005


**Chlorella lowers lipids**


Chlorella and the intestinal tract (colitis, IBS, metal binding)


Nucleotide (CGF/CVE chlorella extracts) und IBS

*Dancey CP*, *Attree EA*, *Brown KF*


*Sporopollein from chlorella attaches itself to toxins in the intestinal mucosa and is excreted in this form together with the toxin* (ref 233 pg 96): Pore, RS – *Drug Chem Toxicol*, 1984;7(1):57-71

**Use of chlorella for toxin elimination**


mercury vapor analyzer is used to show that “nanonized” chlorella (Matrix Metals) and regular chlorella bind mercury

Papers in English by D. Klinghardt, MD,PhD, containing definitive references about the use of chlorella in a detoxification programme:


Klinghardt, D Lecture at the ETH in Zurich: Heavy Metal Toxicity Update (with full discussion on chlorella), Oct 31, 2001 –available on video

Reference about the gold and mercury detoxifying property of chlorella in the book

“The Biosorption of Heavy Metals”; Volesky B, CRC press 1990

Chlorella References, unsorted


15 Kanazawa Medical College Dept. of Serology: *Effects of various preparation*
made from chlorella pyrenoidosa cells on the defence mechanism (immune Resistance), 66-70, 1980.


34 Mündliche Mitteilung während der 3rd International Exhibition & Conference of Vitafoods International, 03.-05.05.2000 in Genf, Schweiz


66 Miner, J.A.: Mechanisms for nutritional inhibition of carcinogenesis in: Moon, T.E., Micozzi, M.S. (Hrsg.): *Nutrition and cancer*


69 Dokumentation der besonderen Therapieeinrichtungen und natürlichen Heilweisen in Europa, Bd. 5, Halbbd. 1, ZDN, Zentrum zur Dokumentation für Naturheilverfahren e.V. (im Auftrag des niedersächsischen Ministerium für Wirtschaft, Technologie und Verkehr), 1992


G. Bartonella
Click album below for photos of Bartonella rashes [http://www.lymediseaseassociation.org/index.php/resources/medical-photos/category/27-bartonella]


*Bartonella henselae* is associated with heartburn, abdominal pain, skin rash, mesenteric adenitis, gastritis and duodentis in children and adolescents.

**H. “Bell’s Palsy of the Gut” and Other GI Manifestations of Lyme and Associated Diseases**

Virginia T. Sherr, M.D., DLFAPA, Distinguished Life Fellow of the American Psychiatric Association, Solo Private Practice of Medicine, Holland, PA.

Virginia T. Sherr

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*Bell’s palsy* signifies paralysis of facial muscles related to inflammation of the associated seventh Cranial Nerve. Physicians may not realize that this syndrome is caused by the *spirochetal agent of Lyme disease until proven otherwise*. Whether it is a full or hemifacial paralysis, Bell’s palsy is cosmetically disfiguring when fully expressed. Sudden loss of normal facial expression terrifies patients who naturally fear they are having a stroke. When a smile is asked for, normal countenances warp into bizarre
grimaces. The amount of tooth area exposed in this attempt to smile helps doctors evaluate the degree of paralysis and its change over time (Figure 1). In every case of Bell’s, doctors need to carefully investigate by history, physical, and laboratory work every shred of evidence that might suggest the presence of cryptic tertiary Lyme, a serious multisystem, gut a patients have no evidence whatsoever of having had a tick-bite.

_Gastrointestinal_ Lyme disease may cause gut paralysis and a wide range of diverse GI symptoms with the underlying etiology likewise missed by physicians. _Borrelia burgdorferi_, the microbial agent often behind unexplained GI symptoms—along with numerous other pathogens also contained in tick saliva—influences health and vitality of the gastrointestinal tract from oral cavity to anus. Disruptions caused by GI borreliosis (Lyme) may include, amongst many others, distortions of taste, failure of other neural functions that supply the entire GI tract—paralysis or partial paralysis of the tongue, gag reflex, esophagus, stomach and nearby organs, small and/or large intestines

**INTRODUCTION** Until proven otherwise, a patient’s unexplained facial paralysis is caused by the tick-borne spirochetes of Lyme disease (LYD) (1). The widely endemic bacteria are easily capable of inducing distal inflammation of the Seventh Cranial (Facial) Nerve (2). “Considering the incidence of Bell’s palsy in Lyme, it is improper to treat it as viral in origin without a work-up for Lyme disease” (3). In an early study with nearly 1000 LYD cases studied, Bell’s palsy occurred in at least 10% of validated cases (4). The frequency of Lyme’s Bell’s palsy etiology is unfamiliar to many physicians. Likewise many physicians are unfamiliar with the spirochetal cause of paralyses of muscles that facilitate normal gastrointestinal transit. Yet, these vital muscles also may be greatly compromised by the same offending neurotropic spirochete, _Borrelia burgdorferi (Bb)_ in patients who are totally unaware of having Lyme disease. Their physicians are often
surprised to learn that persistent Lyme disease is outstandingly a
disease of the brain as well as involving one or all components and
sub-systems of the entire (“ileus”), bowel pseudo-obstruction,
intestinal spasms, excitability of gut muscles,
inflammation of lumen lining tissues, spirochetal
hepatitis, possibly cholecystitis, dysbiosis,
jejunal or ileal incompetence with resultant small
intestine bacterial overgrowth (SIBO), megacolon,
encopresis and rectal muscle cramping (proctalgia fugax).
In cerebral hypothalamic and pituitary centers, usual sites
of borrelial disruptions of the brain’s normal hormonal
cascades, there are strong influences on human attitudes,
ideation, and behavior relating to gastronomic issues.
Newly discovered Lyme-endangered cerebral hormones
and renegade cytokines regulate brain-gut interactions
thus initiating behavioral tendencies such as anorexia or a
failure of satiety with resultant
obesity. Ticks and other vectors of Lyme disease attract
their own infections from many microbes, some known
and some unknown (viruses, amoebas, bacteria, and
possibly parasitic filaria), which they then also can pass
on to humans. The GI tract is especially vulnerable to
machinations of such co-infections as bartonellosis,
mycoplasmosis, human anaplasmosis (HA), and human
monocytic ehrlichiosis (HME). Syndromes exactly similar
to Irritable Bowel Syndrome (IBS), Crohn’s Disease, and
cholecystitis, for example, may not have readily suggested
a borrelial etiology to the diagnostician
but Lyme increasingly is known to be a potential
contributor to each.
All known Lyme-gut syndromes are treated by combining
several effective antimicrobials (including use of azole
medications with specific antibiotics) with agents that
boost gut lining repairs and overall immunity
enhancement. Azole medications are borreliacidal
(against the anti-\textit{Bb} spirochetal cyst form) medications such as metronidazole (Flagyl). Needed GI healing agents may include gut stimulants or relaxants, Ph agents, bile salts, nutriceuticals, immunity-enhancers, neurotoxin absorbents, and sterilizers of gut-specific microbes. Parallelism between Lyme borreliosis-caused paresis of facial muscles supplied by Cranial Nerve VII and Lyme-caused gastrointestinal paralyses suggested a pseudonym to the author—\textit{Bell’s palsy of the Gut}—despite the fact that these syndromes are related to different types of neural fibers and only occasionally occur together. Since similar injury to all sites may be etiologically related, however, otherwise unexplained gastrointestinal symptoms should be considered as possibly related to Lyme borreliosis and/or its co-infections until proven otherwise.

\textbf{References}

5. Nichols TW, Pearce LA. Lyme gastroparesis suggestive of inflammatory neuropathy. Abstract presentations—14th Meeting of the American Motility Society and UICM (9-22 to 25-2005,


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(continued on page 91)

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“Bell’s Palsy of the Gut”


21. Eskow E, Rao RV, Mordechai E. Concurrent infection of the central nervous system by Borrelia burgdorferi and Bartonella
henselae: evidence for a novel tick-borne disease complex. 
22. Los Angeles County West Vector & Vector Borne Disease 
 Bartonella.htm [3-20-2006]
henselae is 
associated with heartburn, abdominal pain, skin rash, 
mesenteric 
adenitis, gastritis and duodenitis. J Pediatr Gastroenterol 
Nutr, 
2002; 35:3. [Abstract #158.]
burgdorferi, 
Bartonella spp., Babesia microti, and Anaplasma 
phagocytophila 
in Ixodes scapularis ticks collected in Northern New Jersey. 
25. Fried MD, Adelson ME, Mordechai E. Simultaneous 
gastrointestinal 
infections in children and adolescents. J Practical 
Gastroenterology, 2004; 78-81. Bartonella rashes: 
http://www.
lymediseaseassociation.org/PhotoAlbum_RashBart.html
disease”-associated arthropathy: don’t forget ticks. Arthritis 
Rheum, In press.
27. Seah ABH, Azran MS, Rucker JC. Magnetic resonance 
imaging 
abnormalities in cat-scratch disease encephalopathy. Journal 
of 
28. Fleisher AS. Case 14: Headache and unilateral visual 
changes. 
Clinical Cases from Johns Hopkins Neurology. Medscape 
Neurology 


31. Medical Diagnostic Laboratories, L.L.C, 2439 Kuser Road, Hamilton, NJ 08690 USA. http://www.mdlab.com/html/testing/available_tests.html#tick


34. IGeneX, Inc. 795 San Antonio Rd., Palo Alto, CA 94303 http://www.igenex.com/about.htm


43. Genova (Great Smokies) Laboratory: http://www.gsdl.com/home/

44. Doctors’ Data Laboratory http://www.doctorsdata.com/home.asp

55. Fallon BA, Nields JA. Lyme disease: a neuropsychiatric illness,
62. Zaidel O, Lin HC. Uninvited guests: the impact of small intestinal

(continued from page 88)

J. Biofilm References

5. The Lyme-Autism Connection: Unveiling the Shocking Link Between Lyme Disease and Childhood Developmental Disorders Paperback, Tami Duncan, Bryan Rosner


I. Depression and Infection

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Reconceptualizing major depressive disorder as an infectious disease

Turhan Canli1,2,3,4

Background

Despite decades of substantial research efforts, major depressive disorder (MDD) remains among the most common mental disorders, with a 16.6%
lifetime prevalence rate [1]. Pharmacological treatment approaches have not changed during this period, targeting primarily receptor-ligand interactions [2]. These types of antidepressants may bring relief to patients with severe symptoms but are not clinically more effective than placebos in mild to moderate cases [3]. Indeed, recurrence rates of 50% for first-episode patients and of 80% for second-episode patients [4] suggest that the core of the illness goes untreated.

Given this track record, I argue that it is time for an entirely different approach. Instead of conceptualizing MDD as an emotional disorder, I suggest to reconceptualize it as some form of an infectious disease. I propose that future research should conduct a concerted search for parasites, bacteria, or viruses that may play a causal role in the etiology of MDD. I present three arguments why this may be a fruitful endeavor. I have outlined the idea in much greater detail elsewhere [5], but will highlight some key points here.

**Main text**

My first argument is that patients with MDD exhibit sickness behavior. Patients experience loss of energy; they commonly have difficulty getting out of bed and lose interest in the world around them. Although our Western conceptualization puts affective symptoms front-and-center, non-Western patients who meet DSM criteria for major depression report primarily somatic symptoms [6-11], reflecting in part cultural differences in the stigmatization of mental illness.

Yet, studies of inflammatory biomarkers in major depression strongly suggest an illness-related origin. For example, a meta-analysis of 24 studies confirmed prior reports of elevated TNFα and IL-6 in patients with major depression [12]. A second meta-analysis of 29 studies further extended the list of significantly elevated inflammatory markers to also include the soluble interleukin-2 receptor [13].

Several postmortem studies report the presence of inflammatory markers in the brains of depressed or mood-disordered patients. For example, compared to controls, female suicide victims showed elevated levels of IL-4 and male suicide victims showed elevated levels of IL-13 in Brodmann Area (BA) 11 [14], a brain region previously associated with suicidal ideation [15,16]. Compared to age-matched controls, patients diagnosed with major depression showed elevated levels of transmembrane TNFα (tmTNFα) in BA46 [17], a region associated with emotion regulation [18-
Patients with major depression, relative to controls, showed differential expression of a large set of both anti- and pro-inflammatory markers (including IL1α, 2, 3, 5, 8, 9, 10, 12A, 13, 15, 18, and IFNγ) in BA10 [21], a region associated with reward processing [22].

These inflammatory markers may represent activation of the immune system in response to some kind of pathogen, which could be a parasite, bacterium, or virus, and which could play a causal role in the etiology of depression. There is currently no direct evidence that major depression is caused by such microorganisms, but nature has offered some examples to illustrate that such a process is conceivable.

Thus, my second argument is that nature has already provided examples by which parasites, bacteria, or viruses can affect emotional behavior. The best-known example of a parasite that affects emotional behavior and that is relevant to human health is *Toxoplasma gondii*. *T. gondii* lives in the feline intestinal tract, where it lays its eggs, which are dispersed into the environment upon excretion. When a rat comes in contact with these eggs and becomes infected, it becomes attracted to the scent of cat urine [23,24]. This manipulation of the rat’s behavior involves the deposit of parasitic cysts across the rodent brain including the amygdala [25]. The mechanism for loss of fear to the scent of cat urine appears to involve a reduction in circulating corticosterone and dendritic retraction in the basolateral amygdala [26]. The mechanism for the rat’s attraction to the odor may involve activation of sexual arousal pathways [27].

The specificity of the behavioral change in the rat’s behavior appears to reflect functional changes that are limited to catecholaminergic neurons [28]. Infected animals have elevated levels of dopamine [29], but *T. gondii* can only synthesize tyrosine hydroxylase (which converts tyrosine to L-DOPA), and would therefore need to rely on catecholaminergic neurons to provide the needed DOPA decarboxylase to convert the L-DOPA to dopamine.

Human exposure to *T. gondii* is pervasive, with one-third of the world’s population [30] and one-fifth of the U.S. population [31] believed to be infected. Infection is associated with elevated inflammatory cytokines IL-6, IL-12, TNF, and IFN-γ [32,33], similarly as observed in depressed patients. A study of 20 European countries reported a positive correlation between *T. gondii* prevalence rates and national suicide rates [34]. Among patients with diagnosed major depression or bipolar disorder, those with a history of suicide attempt had higher *T. gondii* antibody titers [35]. Yet,
large-scale studies of major depression and *T. gondii* or systematic searches to discover other potential parasitic infections have not yet been conducted.

Bacteria could be another causal factor for major depression. Studies of bacterial colonies residing in the gastrointestinal tract have begun to examine links to emotional behavior. In the first study of this kind, germ-free (GF), specific pathogen-free (SPF), and gnotobiotic mice were compared in their response to restraint stress [36]. GF mice exhibited higher levels of plasma ACTH and corticosterone and had lower levels of brain-derived neurotrophic factor in the cortex and hippocampus, compared to SPF mice. The elevated stress response of GF mice was normalized with administration of the bacterium *Bifidobacterium infantis*. Another rodent study showed that administration of *B. infantis* in rats reduced the levels of IFN-γ, TNF-α, and IL-6 following mitogen stimulation and altered tryptophan, 5-HIAA, and DOPAC levels in the frontal cortex and amygdala [37]. Administration of the *Lactobacillus rhamnosus* strain in mice was shown to alter GABAergic expression in the brain: elevating GABA<sub>B1b</sub> mRNA in the cingulate and prelimbic cortices, while reducing it in hippocampus and amygdala, among other regions [38].

The ‘leaky gut’ hypothesis proposes a mechanism by which gastrointestinal bacteria may contribute to major depression [39,40]. According to this hypothesis, cytokines or other stressors may render the intestinal tract permeable to lipopolysaccharides (LPS) from gram-negative bacteria to activate the immune system. Indeed, the model is supported by data showing elevated serum concentrations of IgM and IgA against LPS of the gram-negative enterobacteria in depressed patients [39,40]. These studies were conducted with relatively small numbers of patients and suggested that this mechanism may apply to some subgroups of patients but not others. It would be useful to expand the search using large patient cohorts and a wide range of different antibodies. Future work should then examine potential neural mechanisms.

Viruses represent the third pathogenic route in the etiology of major depression. A meta-analysis of 28 studies explicitly examined the link between infectious agents and depression [41]. Among viruses that had significant associations with the illness were the Borna disease virus (BDV), herpes simplex virus-1, varicella zoster virus, and Epstein-Barr virus. Among these, BDV has been studied most extensively and was 3.25 times more likely to be found in depressed patients than in normal controls [41]. One postmortem study reported BDV infection in 2 out of 30
depressed patients in the frontal and temporal cortex, olfactory bulb, and hippocampus [42], although a larger study failed to detect any infection [43]. A small open-label study of BDV-infected depressed patients reported a reduction in both depressive symptoms and BDV infection upon treatment with the antiviral drug amantadine [44].

The mechanism between BDV infection and depression could involve glutaminergic transmission, because amantadine is an antagonist of the N-methyl-D-aspartate (NMDA) receptor, one of the receptors targeted by glutamate. The related NMDA antagonist memantine has been evaluated in a randomized, double-blind study of patients diagnosed with bipolar depression, where it was applied to augment treatment with the presynaptic glutamate release inhibitor lamotrigine, and found to accelerate treatment response [45]. Another NMDA receptor antagonist, ketamine, also has antidepressant effects [46], which appear to be mediated by changes in mTOR signaling [47]. However, the literature on BDV infection and depression remains controversial, with several studies failing to replicate any association between the two [48-51].

My third argument is that reconceptualizing major depression as being causally linked to parasites, bacteria, or viruses is useful when thinking about the genetics of this illness. Evidence from twin studies notwithstanding, the search for specific genes linked to major depression has come up empty [52,53]. Perhaps, we have been looking at the wrong organism. Genetic studies to date have focused the search on human genes within our genome. Yet, 8% of the human genome is based on exogenous sequences from retroviruses [54]. These retroviral insertions may sometimes benefit the human host and therefore be protected from mutational degeneration [55]. Indeed, the BDV discussed earlier inserted some of its sequences into vertebrate genomes approximately 40 million years ago [56], and presence of these sequences correlates with disease resistance to BDV. Parasites could also add exogenous sequences to the human genome through the process of horizontal gene transfer [57]. It is possible that polymorphisms within such exogenous sequences, or interactions between these exogenous sequences and other variables such as human gene polymorphisms or stressful life experiences, could render some individuals vulnerable to major depression.

Furthermore, if we view the human body as an ecosystem that is a host to numerous microorganisms which may be passed across generations, the opportunity for genetic discoveries is vastly amplified. For example, an estimated thousand species of bacteria reside in the human gastrointestinal
tract [58], and these could be passed during birth or through common environmental exposure between parents and offspring [59]. Humans also carry vast numbers of viruses, which can be unknown and go undetected until subjected to a concerted search using new approaches such as deep sequencing [60].

Conclusions

In light of the above considerations, an important point of reflection concerns the relation between the immune response and MDD and the specificity of any putative mechanism. The literature implicating the immune system in MDD [61] can be read as suggesting that the immune response itself is the causal mechanism in depression. Indeed, conditions such as hypoxia known to produce sterile inflammation ([62], i.e., activation of the immune system sans a pathogen) may increase the risk of depression [61] in conditions such as obstructive sleep apnea [63] or chronic obstructive pulmonary disease [64]. Yet, most cases of MDD are not attributable to sterile inflammation. Thus, I suggest that some unknown pathogen(s) could play a causal role, and that the immune response is secondary to the infection; interventions that only target the immune response may bring symptom relief but would not address the root cause of the illness.

If a pathogen played a causal role in MDD, the next question would concern the specificity of the mechanism. One perspective would favor a very general, non-specific mechanism. For example, chronic fatigue syndrome (CFS)—which is characterized by sickness behavior that may include depressive symptoms—has been hypothesized to be caused by vagus nerve infection, regardless of the type of pathogen [65]. My view is that, for MDD, the type of pathogen may matter a great deal, and that it plays a very specific causal role: the examples I presented above suggest plausible mechanisms by which pathogens may alter neurotransmission. However, there may not be a single pathogen that causes all cases of MDD. Instead, there may be a class of pathogens, similar to those discussed above, which share common modes of action. This class of pathogens would specifically target the nervous system in a manner that causally contributes to MDD. I use the term ‘contribute’ to indicate that these pathogens may act in concert with other variables. For example, an individual may carry a latent infection and be asymptomatic for depressive symptoms. This individual would be characterized by susceptibility to
MDD which may only emerge after the pathogen was activated by other factors such as stressful life events; this activation could then also trigger a concomitant immune response. It is possible that such a pathogen-driven mechanism is not limited to MDD but may contribute to other psychopathologies. For example, posttraumatic stress disorder could be one such extension of the same mechanism: not every individual develops the disorder in response to a traumatic experience (suggesting individual differences in susceptibility), and the illness is accompanied by immune system activation [66,67].

In closing, I think it would be worthwhile to conduct large-scale studies of carefully characterized depressed patients and healthy controls, using gold-standard clinical and infectious disease-related study protocols, as have already been developed for bacteria [68,69] and viruses [70-76]. Such efforts, if successful, would represent the ‘end of the beginning’, as any such discovery would represent the first step toward developing a vaccination for major depression.

**Abbreviations**

BA: Brodmann Area; BDV: Borna disease virus; GABA: gamma-aminobutyric acid; IFN-γ: interferon gamma; IgA: immunoglobulin A; IgM: immunoglobulin M; IL: interleukin; L-DOPA: L-3,4-dihydroxyphenylalanine; LPS: lipopolysaccharides; MDD: major depressive disorder; NMDA: N-methyl-D-aspartate; TNFα: tumor necrosis factor alpha; tmTNFα: transmembrane tumor necrosis factor alpha.

**Competing interests**

The author declares that he has no competing interests.

**References**


31. Centers for Disease Control and Prevention (CDC) Parasites - toxoplasmosis (toxoplasma infection) [http://www.cdc.gov/parasites/toxoplasmosis/epi.html]


45. Stevens J, Bies RR, Shekhar A, Anand A. Bayesian model of Hamilton depression rating score (HDRS) with memantine


47. Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science. 2010;329:959–964. doi: 10.1126/science.1190287. [PMC free article] [PubMed][Cross Ref]


61. Dantzer R, O’Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci. 2008;9:46–56. doi: 10.1038/nrn2297. [PMC free article] [PubMed] [Cross Ref]


Healing Lyme with Antibiotics 2016

ORIGINAL RESEARCH ARTICLE


Eradication of Biofilm-Like Microcolony Structures of Borrelia burgdorferi by Daunomycin and Daptomycin but not Mitomycin C in Combination with Doxycycline and Cefuroxime

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Lyme disease, caused by Borrelia burgdorferi, is the most common vector-borne disease in the United States and Europe. While the majority of Lyme disease patients can resolve their symptoms if
treated promptly, 10–20% of patients suffer from prolonged symptoms called post-treatment Lyme disease syndrome (PTLDS). Although the cause for PTLDS is unclear, one possibility is the presence of bacterial persisters not effectively cleared by the current Lyme antibiotics. Recent studies identified several drug candidates including daptomycin, daunomycin, doxorubicin, and mitomycin C that had good activity against *B. burgdorferi* persisters. However, their relative activities against *B. burgdorferi* persisters have not been evaluated under the same conditions. In this study, we tested the anti-persister activities of these drugs against both 7-day and 15-day old stationary phase cultures of *B. burgdorferi* individually as well as in combination with Lyme antibiotics doxycycline and cefuroxime (Ceftin). Our findings demonstrate daunomycin and daptomycin were more active than mitomycin C in single drug comparison at 10 and 20 µM, as well as in drug combinations with doxycycline and cefuroxime. In addition, daunomycin was more active than doxorubicin which correlated with their ability to stain and accumulate in *B. burgdorferi*. The two drug combination of doxycycline and cefuroxime was unable to eradicate biofilm-like microcolonies of *B. burgdorferi* persisters. However, the addition of either daunomycin or daptomycin to the doxycycline + cefuroxime combination completely eradicated the biofilm-like structures and produced no visible bacterial regrowth after 7 and 21 days, while the addition of doxorubicin was unable to prevent regrowth at either 7 or 21 day subculture. Mitomycin C in combination with doxycycline and cefuroxime caused no regrowth at 7 days but visible spirochetal regrowth occurred after 21 day subculture. Furthermore, we found that cefuroxime (Ceftin), the third commonly used and most active antibiotic to treat Lyme disease, could replace cefoperazone (a drug no longer available in the US) in the daptomycin + doxycycline combination with complete eradication of the biofilm-like structures as shown by lack of any regrowth in subcultures. Our findings may have implications for improved treatment of Lyme disease.

**Introduction**

*Borrelia burgdorferi* is the causative agent of Lyme disease, which is the most common vector-borne disease in the United States with an estimated 300,000 cases in 2013 ([CDC, 2015a](https://www.cdc.gov/). The infection is transmitted to
humans by tick vectors that feed upon rodents, reptiles, birds and deer (Radolf et al., 2012). In the early stage of Lyme disease, approximately 50% of patients have localized erythema migrans, a target-shaped rash that expands as the bacteria disseminate from the cutaneous infection site (CDC, 2015a). Late stage Lyme disease is a multi-system disorder with symptoms including arthritis, carditis, and neurologic impairment (CDC, 2015a). The majority of Lyme disease patients can resolve their symptoms if treated promptly with doxycycline, amoxicillin, or cefuroxime (Wormser et al., 2006). However, at least 10–20% of Lyme disease patients experience prolonged symptoms such as neurologic impairment, muscular pain, and fatigue 6 months after antibiotic treatment, a collection of symptoms called Post-Treatment Lyme Disease Syndrome (PTLDS; CDC, 2015b).

The cause of PTLDS is unknown, though there are several theories including co-infections (Swanson et al., 2006), autoimmune response (Steere et al., 2001), immune response to continued presence of antigenic debris (Bockenstedt et al., 2012), as well as B. burgdorferi persisters that are not killed by the current antibiotics (Hodzic et al., 2008, 2014; Embers et al., 2012). Using a combination of diagnostic techniques including xenodiagnosis and PCR, studies have found evidence of B. burgdorferi persistence in dogs (Straubinger et al., 1997), mice (Hodzic et al., 2008, 2014), monkeys (Embers et al., 2012), and humans (Marques et al., 2014) after antibiotic treatment, though no viable bacteria could be cultured.

*Borrelia burgdorferi* develops persisters stochastically in stationary phase which are tolerant to the antibiotics used to treat Lyme disease (Feng et al., 2014a, 2015a; Caskey and Embers, 2015; Sharma et al., 2015). These persister bacteria have been found to have an altered RNA profile, making them phenotypically drug tolerant (Feng et al., 2015c). In log phase cultures (3–5 days old), *B. burgdorferi* is primarily in motile spirochetal form which is highly susceptible to current Lyme antibiotics doxycycline and amoxicillin, however, in stationary phase cultures (7–15 days old), increased numbers of atypical forms such as round bodies and aggregated
biofilm-like microcolonies develop (Feng et al., 2014a, 2015a). These atypical forms have been shown to have increased tolerance to doxycycline and amoxicillin when compared to the growing spirochetal forms (Feng et al., 2014a, 2015a; Caskey and Embers, 2015; Sharma et al., 2015). Therefore, stationary phase cultures (7–15 days old) which are enriched in persisters were used as a model for high-throughput drug screens against persister populations (Feng et al., 2014a, 2015a,b,d).

Drugs with high activity against the B. burgdorferi stationary phase persisters were identified through screens of FDA approved drug library and NCI compound libraries (Feng et al., 2014a, 2015b,d). Among them, daptomycin, a lipopeptide antibiotic targeting bacterial cell membranes, was found from the FDA drug library to have the highest anti-persister activity against B. burgdorferi (Feng et al., 2014a). In addition, anticancer anthracycline antibiotics, such as daunomycin and doxorubicin, and also mitomycin C were found from the NCI compound library screen as having excellent or good activity against B. burgdorferi persisters (Feng et al., 2015b). Daunomycin, doxorubicin and mitomycin C were all isolated from Streptomyces and are used in the treatment of a wide range of cancers. Daunomycin and doxorubicin belong to anthracycline anti-cancer antibiotic and kill the bacteria by inhibiting DNA and RNA synthesis, causing DNA damage and producing reactive oxygen species. Mitomycin C blocks DNA replication and causes cell death by DNA crosslinking.

Although the anti-persister drugs such as daptomycin are more active than the current Lyme antibiotics such as doxycycline or amoxicillin against B. burgdorferi persisters (Feng et al., 2014a), they alone could not completely eradicate the more resistant biofilm-like microcolonies and a drug combination approach is required to do so (Feng et al., 2015a). The more effective drug combination approach to eradicate biofilm-like microcolonies is consistent with the drug combination principle for treatment of persistent infections like tuberculosis (Zhang et al., 2012; Zhang, 2014). In a recent study using a relatively young 5 days old culture, mitomycin C was found to have higher activity than daptomycin against B.
Burgdorferi persisters (Sharma et al., 2015). However, their relative activity against B. burgdorferi persisters has not been compared or evaluated in the same study under the same conditions. In this study, four of the identified drugs with the highest activity against stationary phase B. burgdorferi persisters were tested to determine their anti-persister activity at more clinically achievable levels. In addition, we assessed these persister active drugs in combination with the commonly prescribed Lyme antibiotics doxycycline and cefuroxime, which have high activity against growing log phase cultures, with the aim to increase the activity of these drugs for more effective treatment of Lyme disease.

**Materials and Methods**

**Strain, Media, and Culture Techniques**

*Borrelia burgdorferi* strain B31 (ATCC 35210) was obtained from American Type Tissue Collections (Manassas, VA, USA). *B. burgdorferi* was grown in BSK-H medium (HiMedia Laboratories, Mumbai, India) and supplemented with 6% rabbit serum (Sigma Aldrich, St. Louis, MO, USA). The medium was filter-sterilized via passage through a 0.22 µM filter. The inoculated medium was incubated in sterile 50 mL conical tubes (BD Biosciences, San Jose, CA, USA) in a 33°C incubator without shaking. The culture was maintained in these conditions for 7 or 15 days until the culture reached stationary phase, when it was transferred to a 96 well plate for evaluation with the drugs or their combinations.

**Drugs**

The following drugs were obtained from Sigma–Aldrich, St. Louis, MO, USA and dissolved in the solvents suggested by the Clinical and Laboratory Standards Institute to make a stock solution: doxycycline (Dox), cefuroxime (CefU), cefoperazone (CefP), daptomycin (Dap), mitomycin C (MitC), doxorubicin (DoxR), daunomycin (Dau), (Clinical and Laboratory Standards Institute, 2007). The drug stock solutions were filter-sterilized using a 0.22 µM filter and stored at –20°C.

**Microscopy**
The *B. burgdorferi* cultures were examined using a Zeiss AxioImager M2 microscope with differential interference contrast and epifluorescent illumination. Pictures were taken using a SPOT slider camera. A SYBR Green I/PI assay was performed as previously described to assess cell viability using the ratio of green:red fluorescence to determine the live:dead cell ratio, respectively, as measured by a plate reader (Feng et al., 2014b). This residual cell viability reading was confirmed by analyzing three representative images of the bacterial culture using epifluorescence microscopy. Image Pro-Plus software was used to quantitatively determine the fluorescence intensity.

**Evaluation of Drugs Against Biofilm-Like Structures in *B. burgdorferi* Stationary Phase Cultures**

For single drug evaluation, an aliquot of the drug stock solution was added to each 96 well plate containing 100 µL of 7-day old stationary phase *B. burgdorferi* culture to obtain the desired drug concentration. The plate was then sealed and was incubated at 33°C without shaking for 7 days. After incubation, the viability of the residual viable cells was assessed using the SYBR Green I/PI viability assay and confirmed using epifluorescence microscopy (Feng et al., 2014b). Each sample was analyzed in triplicate and the mean residual viable cells remaining were calculated.

For assessing the activity of anthracycline compounds and daptomycin and mitomycin C in combination with current Lyme antibiotics against biofilm-like structures, a 15-day old *B. burgdorferi* stationary phase culture was used. Aliquots of the drugs were added to 96 well plate containing 100 µL of the 15-day old stationary phase *B. burgdorferi* culture which was enriched in aggregated biofilm-like structures to create a final drug concentration of 10 µg/mL for each drug. This drug concentration was chosen as most drugs evaluated in this study fell within or close to their $C_{\text{max}}$ values (maximum serum concentration; Table 1). The plate was then sealed and was incubated at 33°C without shaking for 7 days, when the residual viable cells remaining were measured using the SYBR Green I/PI viability assay and confirmed using epifluorescence microscopy as described (Feng et al., 2014b).
TABLE 1. Relative activity of daunomycin, daptomycin, doxorubicin, and mitomycin C on a 7-day old B. burgdorferi stationary phase culture.

**Subculture Study to Assess the Effect of Drug Combination on the Biofilm-Like Structures in B. burgdorferi Stationary Phase Cultures**

A 15-day old B. burgdorferi culture (500 µL of $1 \times 10^7$ spirochetes/mL) was exposed to the indicated drug combinations in Eppendorf tubes, and incubated at 33°C for 7 days without shaking. After incubation, the bacteria were spun down and washed with 1 mL fresh BSK-H medium. The cultures were resuspended in 500 µL BSK-H medium, and a 50 µL aliquot was used to inoculate a new tube of 1 mL fresh BSK-H medium for subculture. The cultures were allowed to grow for either 7 or 21 days, when they were evaluated for regrowth with viable cells using the previously described SYBR Green I/PI assay and epifluorescence microscopy (Feng et al., 2015a).

**Results and Discussion**

**Comparison of the Relative Anti-Persister Activity of Daunomycin, Doxorubicin, Daptomycin, and Mitomycin C in Single Drug Exposure Against Stationary Phase B. burgdorferi Culture**

Although daptomycin (Feng et al., 2014a), daunomycin (Feng et al., 2015b), doxorubicin (Feng et al., 2015b), and mitomycin C (Feng et al., 2015b; Sharma et al., 2015) were identified to have high activity against B. burgdorferi persister, their relative activities have not been compared under the same conditions. To do so, we compared them for their activity against the same 7-day old B. burgdorferi stationary phase culture at the same concentrations (5, 10, and 20 µM), using SYBR Green I/PI viability
stain followed by epifluorescence microscopy. The anthracycline drug daunomycin was shown to have the highest activity against the stationary phase cultures even at the lowest concentration (5 µM) with a dose-dependent increase in killing activity resulting in a near total clearance of bacteria at the highest concentration (20 µM) as shown by mostly red (dead) cells and dispersed, smaller aggregated microcolony size, revealed by the SYBR Green I/PI viability assay (Figure 1, Table 1). Daptomycin was the second most active drug against the B. burgdorferi stationary phase culture, followed by doxorubicin (Figure 1, Table 1). Mitomycin C was the least active drug among the four persister-active drugs, and even at 20 µM had poor activity against the aggregated biofilm-like microcolony form of B. burgdorferi persisters, as shown by mostly green (live) microcolonies remaining after the drug treatment for 7 days (Figure 1).

FIGURE 1

A 7-day old B. burgdorferi stationary phase culture containing aggregated microcolonies was incubated for 7 days with daptomycin (Dap), daunomycin (Dau), doxorubicin (DoxR), or mitomycin C (MitC) at the same drug concentrations of 5, 10, or 20 µM, respectively, followed by viability assessment using the SYBR Green I/PI assay. Representative images were taken using epifluorescence microscopy at 400× magnification. Green cells indicate live cells while red cells indicate dead cells.
Doxorubicin was less active than daunomycin as shown by higher percentage of viable cells remaining after drug exposure (Table 1) despite their both belonging to the same anthracycline class. These results could be explained by structural differences between those compounds (Figure 2A). Doxorubicin possesses a hydroxyl group as opposed to a methyl group in the corresponding position of daunomycin, with the remainder of the anthracycline structure being identical. Interestingly, although doxorubicin and daunomycin both have orange-red color in solution (Figure 2B), we found daunomycin visibly stained the *B. burgdorferi* cells red as seen in the red cell pellet while doxorubicin only stained the cells rather faintly (Figure 2C). This finding suggests that daunomycin may cross the *B. burgdorferi* cell membrane more efficiently to accumulate in the cell while doxorubicin may have poor ability to enter or accumulate in *B. burgdorferi* cells.

**FIGURE 2**

**FIGURE 2.** Differences in structures of daunomycin and doxorubicin and their ability to accumulate in *B. burgdorferi*. (A) Chemical structures of daunomycin and doxorubicin. Red box shows the difference between the structures of daunomycin (methyl group) and doxorubicin (hydroxyl group). (B) Daunomycin and doxorubicin show the same orange–red color at 10 mM solution. (C) Cell pellets of 7-day old *B. burgdorferi* treated with 10 µM daunomycin (left-side tube) 10 µM doxorubicin (right-side tube) for 7 days, where daunomycin stained *B. burgdorferi* red while doxorubicin hardly stained the organism.

**Comparison of the Relative Anti-Persister Activity of Daunomycin, Daptomycin, Doxorubicin, and**
Mitomycin C in Drug Combinations Using SYBR Green I/PI Viability Assay

Both two-drug combinations doxycycline + cefuroxime and doxycycline + cefoperazone showed poor activity against the 15-day old stationary phase culture, with 67% residual viable (green) cells remaining in comparison to 79% viable cells in the drug-free control (Figure 3, Table 2). Consistent with the single drug exposure experiment (Figure 1), daunomycin, doxorubicin and daptomycin when added to the drug combination doxycycline + cefuroxime had a high anti-persister activity as seen by 12, 18, and 30% viable cells remaining (Table 2) as well as mostly red (dead) cells after treatment (Figure 3). In contrast, when mitomycin C was added to the drug combination doxycycline + cefuroxime, the anti-persister activity of these compounds was moderately increased as shown by 45% residual viable cells remaining (Table 2), but more green (live) cells were seen with the mitomycin C drug combination than with the daunomycin or daptomycin drug combination (Figure 3).

FIGURE 3

FIGURE 3. Comparison of the activity of daunomycin, daptomycin, and mitomycin C in combination with currently used Lyme antibiotics. A 15-day old B. burgdorferi stationary phase culture was incubated with the indicated drug combinations at a final concentration of 10 µg/mL for each antibiotic for 7 days followed by SYBR Green I/PI stain and epifluorescence microscopy. Abbreviations: Dox, doxycycline; CefU, cefuroxime; CefP, cefoperazone; Dap, daptomycin; MitC, mitomycin C; DoxR, doxorubicin; Dau, daunomycin.

TABLE 2
TABLE 2. Viability of stationary phase *B. burgdorferi* after antibiotic treatment assessed by direct SYBR Green I/PI viability assay and subculture.

Subculture Study to Assess the Relative Anti-Persister Activity of Daunomycin, Daptomycin, and Mitomycin C in Drug Combinations

To validate the activity of these drug combinations, samples of the above drug-treated cultures were subjected to subculture after removal of the drugs by washing followed by incubation in fresh BSK medium for 7 or 21 days. A lack of visible regrowth when measured by microscopy suggests that few to no viable cells remain after drug treatment, while visible regrowth of the culture indicates the presence of viable cells after drug treatment. The addition of daunomycin or daptomycin to the doxycycline + cefuroxime drug combination showed no visible regrowth after 7 and 21 days, suggesting no viable *B. burgdorferi* organisms were left after the treatment (Figure 4). Despite the high anti-persister activity of doxorubicin + doxycycline + cefuroxime in the microscopic analysis (Figure 3), with only 18% residual viable cells after treatment (Table 2), this triple drug combination was unable to prevent bacterial regrowth at either 7 or 21 days subculture, indicating it is not as active as daunomycin or daptomycin (Table 2, Figure 4). However, doxorubicin + doxycycline + cefuroxime was more active than mitomycin C + doxycycline + cefuroxime as shown by less regrowth than the latter combination (Figure 4). This is consistent with the single drug data where doxorubicin was more active than mitomycin C (Figure 1).
FIGURE 4. Subculture (21 days) of 15-day old B. burgdorferi stationary phase culture treated with different drug combinations. The 15-day old B. burgdorferi culture was incubated with the indicated drug combinations at a final concentration of 10 g/mL for each antibiotic for 7 days followed by washing and resuspension of cells in fresh BSK medium and subcultured for 21 days. The viability of the subculture was examined by SYBR Green I/PI viability assay and epifluorescence microscopy (400 magnification). NG, no growth.

The discrepancy in the activity of doxorubicin in epifluorescence microscopy based viability analysis and subculture study was noted in our previous studies (Feng et al., 2015a,b). This is due to the red orange color of the anthracycline drug doxorubicin, which stains the cells red and could give false impression of a high killing activity. However, subculture studies were able to show the inability of doxorubicin + doxycycline + cefuroxime to eradicate the microcolony form of B. burgdorferi persisters as shown by regrowth after subculture. Thus, the subculture study is crucial in validating the results of other forms of viability assays such as SYBR Green I/PI assay in persister drug evaluations.

When mitomycin C was added to doxycycline + cefuroxime combination treated B. burgdorferi stationary phase culture, there was no regrowth at 7 days, but visible spirochetal regrowth occurred after 21 days subculture (Figure 4). This finding suggests that the addition of mitomycin C to the commonly used Lyme antibiotics doxycycline + cefuroxime is not as active as the addition of daunomycin or daptomycin, but is more active than doxorubicin. Furthermore, this result indicates that 7 days subculture is not sufficient to reveal the small number of residual bacteria remaining after drug treatment and that a prolonged incubation to 21 days is needed to demonstrate the small numbers of viable bacteria for more reliable evaluation of drug combinations against B. burgdorferi persisters.
In a recent study, mitomycin C was shown to be more active than daptomycin and to eradicate all *B. burgdorferi* persisters ([Sharma et al., 2015](#)). This is in contrast to the results of this study which found daptomycin to have higher anti-persister activity than mitomycin C in both single drug exposure (Table 1, Figure1) and drug combination studies (Figures 3 and 4). Several possibilities exist to explain the discrepancy. First, we used older 7 and 15 days stationary phase cultures containing an increased number of persisters and biofilm-like microcolonies previously shown to have increased tolerance to antibiotics ([Feng et al., 2015a](#)), while the other study used a younger culture of 5 days ([Sharma et al., 2015](#)), which would have more growing cells and fewer persister cells. The difference in persister numbers in these cultures would result in the bacteria in the younger culture of 5 days being more easily killed by mitomycin C but not by daptomycin. Indeed, daptomycin is known to have relatively high MIC (12.5–25 mg/mL) for growing spirochetes despite its high activity against *B. burgdorferi* persisters ([Feng et al., 2014a](#)), and this may also explain why daptomycin had limited activity in that study as a younger culture was used ([Sharma et al., 2015](#)). The use of a younger culture in the other study that contained mainly growing spirochetes is also consistent with their finding that the 5-day old culture was readily killed by even amoxicillin and ceftriaxone ([Sharma et al., 2015](#)), which are known to kill mainly growing bacteria. Second, we used different viability assays. In this study, we used SYBR Green I/PI viability stain along with microscopy and subculture in liquid medium to assess the viability of residual bacteria after drug treatment. In contrast, the other study used colony forming unit (CFU) assay on solid agar to determine the viable bacteria after drug exposure ([Sharma et al., 2015](#)). Based on studies with other bacteria like *M. tuberculosis* ([Dhillon et al., 2004](#)), the CFU assay favors the detection of more viable organisms and is less sensitive than culture in liquid medium which can detect small numbers of viable cells which may not grow on solid medium after drug exposure. Third, we used BSK-H medium, which is richer than the BSK-II medium used by the other study ([Sharma et al., 2015](#)).
Cefuroxime (Ceftin) Could Replace Cefoperazone in the Daptomycin + Doxycycline Combination to Completely Eradicate Biofilm-Like Structures

In our previous drug combination study, we found that daptomycin + doxycycline + cefoperazone was able to completely eradicate the most resistant aggregated biofilm-like microcolonies (Feng et al., 2015a). However, since cefoperazone is not available in the US, we replaced it with the current Lyme antibiotic cefuroxime (Ceftin) in the daptomycin + doxycycline combination and found they had equivalent activity as shown by the same 30% residual viable cells after antibiotic treatment using SYBR Green I/PI viability stain and microscopy (Figure 3, Table 2). In subculture studies, we found replacement of cefoperazone with cefuroxime (Ceftin) in the daptomycin + doxycycline combination similarly resulted in complete eradication of the biofilm-like structures as shown by lack of any regrowth in 7 and 21 days subcultures (Figure 4).

Cefuroxime and cefoperazone, which are second and third generation cephalosporins, respectively, function as a highly penetrative beta-lactam antibiotic by disrupting the bacterial cell wall biosynthesis (Barriere and Flaherty, 1984). Despite being reported as the best beta-lactam antibiotic in the 7-day stationary phase persister model (Feng et al., 2014a), cefoperazone did not give any advantage over the use of the commonly prescribed Lyme antibiotic cefuroxime (Ceftin) in the context of drug combination with daptomycin + doxycycline (Figures 3 and 4). This data suggests that replacing cefoperazone with the commonly used cefuroxime (Ceftin) will maintain comparable efficacy against the biofilm-like microcolony form of B. burgdorferi persisters in the drug combination with daptomycin + doxycycline.

In this study, we were able to confirm our previous observations of the high anti-persister activity of daptomycin (Feng et al., 2014a, 2015a) and daunomycin (Feng et al., 2015b) alone and in drug combination with
doxycycline + cefuroxime. It is worth noting that the high anti-persister activity of daptomycin and the anthracycline antibiotic daunomycin is due to the unique mechanisms of action through disruption of cell membrane and damage of DNA, respectively (Feng et al., 2014a, 2015b). These observations suggest that bacterial membranes and DNA integrity are important targets for bacterial persister drugs. The complete eradication of biofilm-like structures of B. burgdorferi by daunomycin or daptomycin in drug combination with doxycycline + cefuroxime, again supports the Yin–Yang treatment principle of combining drugs that target growing bacteria (Yang; with doxycycline + cefuroxime) and drugs like daunomycin or daptomycin that target non-growing persisters (Yin) for more effective treatment of persistent infections (Zhang, 2014). This strategy may be generally useful for treatment of persistent infections including biofilm infections, which cannot be eradicated by a single drug alone. Future studies are needed to validate this principle.

Although our findings that daunomycin or daptomycin plus doxycycline + cefuroxime could completely eradicate the biofilm-like structures are encouraging, they are in vitro studies and have limitations and cannot be equated to the clinical situation. Moreover, daunomycin and daptomycin are intravenous drugs and not convenient to administer. Future studies to develop oral regimens as effective as the above combinations are needed for more convenient administration. In addition, the toxicity associated with the anticancer drug daunomycin calls for caution with its use in clinical settings. Further in vivo animal studies are needed to validate the highly active drug combinations identified in this study before they can be used for patient treatment in the clinic.

**Conclusion**

In summary, we found that daunomycin and daptomycin were more active against B. burgdorferi biofilm-like structures than mitomycin C and doxorubicin in single drug comparisons as well as in drug combinations. Daunomycin or daptomycin when added to doxycycline + cefuroxime completely eradicated the biofilm-like structures, while the two drug
combination doxycycline + cefuroxime alone or mitomycin C and
doxorubicin when added to the above combination failed to do so.
Additionally, we showed that cefuroxime (Ceftin) could replace
cefoperazone in the daptomycin + doxycycline combination and caused
complete eradication of the biofilm-like structures. Future studies are
needed to evaluate these promising drug combinations in vivo in animal
models, and if promising, in patients. Our findings may have implications
for improved treatment of Lyme disease.

**Author Contributions**
YZ conceived the experiments; JF, MW, WS, SZ, performed the
experiments; JF, MW, and YZ analyzed the data; and MW, JF, YZ wrote the
paper.

**Conflict of Interest Statement**
The authors declare that the research was conducted in the absence of any
commercial or financial relationships that could be construed as a potential
conflict of interest.

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**References**

[Google Scholar](#)

Bockenstedt, L. K., Gonzalez, D. G., Haberman, A. M., and Belperron, A. A.
(2012). Spirochete antigens persist near cartilage after murine Lyme


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