

## REVIEW

**Innate and procured immunity inside the digestive tract of the medicinal leech****AC Silver<sup>1</sup>, J Graf<sup>2</sup>**<sup>1</sup>*Department of Internal Medicine, Section of Infectious Diseases, Yale University School of Medicine, New Haven, CT 06520, USA*<sup>2</sup>*Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269, USA**Accepted August 29, 2011***Abstract**

Especially when combined with unique biological adaptations, invertebrate animals provide important insights into innate immunity because the immune response is not complicated by adaptive immunity that vertebrates evolved. One such example is the digestive tract of the medicinal leech, *Hirudo verbana*, which is unusual in two aspects, it contains a simple microbial community and it stores large amounts of vertebrate blood for a several months. In this review we will discuss aspects of the innate immunity of the leech and from the ingested blood that we term procured immunity to differentiate it from the immunity encoded by the leech genome.

**Key Words:** medicinal leech; *Aeromonas*; innate immunity**Introduction**

The ability of medicinal leech to consume large quantities of vertebrate blood is a fascinating biological adaptation of this slow moving parasite and the underlying reason for the resurgence of its use in today's surgical wards. In a single blood meal *Hirudo verbana*, the Hungarian medicinal leech, can consume over 5-times its body weight in blood. This process is facilitated by the release of vasodilators and anticoagulants that stimulate the blood flow and activate pumping of the leech (Rigbi *et al.*, 1987, 1996). The anticoagulants are so powerful that the bite site will continue to bleed for about 15 minutes after an engorged leech falls off its prey. While there are many uses of medicinal leeches, one particular use led to the approval of the medicinal leech as a medical device by the FDA (Rados, 2004). During reconstructive microvascular surgery, a major complication is venous congestion (Whitaker *et al.*, 2005; Whitaker *et al.*, 2009). While blood can enter the reattached tissue through the reconnected arteries, the blood does not exit at a sufficient rate, leading to a lack of oxygen and tissue necrosis occurs. Application of medicinal leeches removes blood from the tissue allowing oxygenated blood to enter the wound. This procedure greatly increases the success rate in cases with this complication but also carries the risk of wound infections by one of

the digestive-tract symbionts and thus a preemptive antibiotic therapy is suggested (Whitaker *et al.*, 2011).

Consuming such large blood meals requires several adaptations. The storage occurs in the largest compartment of the digestive tract, the crop, which accounts for most of the animal's body. Water and salts are removed from the ingested blood meal concentrating it into a viscous paste, which allows the animal to regain mobility (Sawyer, 1986; Zebe *et al.*, 1986). Interestingly, it has been reported that at least some of the erythrocytes remain physically intact inside the crop for several months (Jung, 1955), but a recent report indicated that a portion of the erythrocytes are lysed by the digestive tract symbionts (Maltz and Graf, 2011). Over a period of many weeks, the blood meal moves into the intestine where the digestion of the blood meal and absorption of nutrients is thought to occur. The animals can survive for over six months between feedings.

Historically, *Hirudo medicinalis*, the medicinal leech, from central Europe was used for application on humans in France and Germany. A combination of heavy collecting and loss or pollution of habitat led to a decline in the natural populations. This led to *H. medicinalis* being listed as an endangered species and the emergence of leech farms where the animals are raised and also bred in captivity (reviewed in Graf, 2000). Medical supply companies typically sold medicinal leeches as *H. medicinalis* but it was recently discovered that these animals are usually *H. verbana*, the Hungarian medicinal leech (Siddall *et al.*, 2007). It seems likely that most

*Corresponding author:*

Joerg Graf

Department of Molecular and Cell Biology  
University of Connecticut, 91 N. Eagleville Rd  
Unit-3125, Storrs, CT 06269, USA  
E-mail: joerg.graf@uconn.edu

leech farms supplemented their breeding stock with field caught animals from Eastern Europe and thus inadvertently replaced *H. medicinalis* with *H. vervana*. There are some differences in the pigmentation but DNA bar coding is the most useful technique in differentiating these species. Given the paucity of *H. medicinalis* among commercially available animals, it seems reasonable that most research is done on *H. vervana* or perhaps *Hirudo orientalis*.

As all animals, the leech lives in close association with microorganisms that are thought to provide essential function for the leech. In the leech, the digestive tract is colonized by a relatively simple microbial community (Worthen *et al.*, 2006), which may be critical for preventing a premature degradation of the ingested blood meal. Early culture-based studies reported the presence of a single bacterial symbiont that we identified as *Aeromonas veronii* in the crop (Graf, 1999). Subsequently, a culture-independent study revealed the presence of a second numerically dominant bacterial symbiont, a *Rikenella*-like bacterium (Kikuchi and Graf, 2007; Worthen *et al.*, 2006). In the intestine, the same two species are highly abundant, but a greater diversity of species exists. Diagnostic PCR of embryos revealed that the bacteria are transmitted from parent to offspring (Rio *et al.*, 2009). It is very intriguing that a gut community is this simple, even insects that have a highly alkaline midgut harbor a more complex community of microorganisms (Broderick *et al.*, 2004).

A driving question is to determine which factors contribute to this unusual simplicity. One approach to address this question is to experimentally determine how specific the association is and identify mechanisms that are responsible.

### Procured immunity

A colonization assay allows one to assess the ability of different species or mutants to colonize an animal (Graf, 1999). For the medicinal leech, we can introduce the bacteria that carry antibiotic resistance markers into the gut by adding the bacteria to a blood meal. By plating dilutions of the gut content onto antibiotic containing plates we can readily tell them apart from the native bacteria. A simple control provides insight into the changes that occur inside the gut (Indergand and Graf, 2000). An aliquot of the inoculated blood is incubated inside a microcentrifuge tube at the same temperature as the leech. A comparison of the density of colony forming units (CFU), or viable bacteria, allows one to deduce how well a strain is able to grow inside the leech gut and if that growth reduced inside the leech as compared to the blood *in vitro* control or in comparison to a native leech symbiont.

An *Escherichia coli* strain exhibited an interesting phenotype (Indergand and Graf, 2000). For the first 42 h after being fed to the leech, the number of *E. coli* that could be cultured from inside the leech decreased ~100 to 1,000 fold. Interestingly, a similar decrease occurred in the *in vitro* blood control thus it seemed possible that the same activity that killed off the bacteria in the control

could remain active inside the leech. Interfering with the activation of the complement system in the blood meal prior to inoculating it with the *E. coli* strain either with heat, EDTA or EGTA with Mg<sup>2+</sup> permitted the *E. coli* strain to proliferate in blood, but in blood supplemented with inulin, the *E. coli* strain did not grow. This suggested that interfering with the activation of the complement system, perhaps via the classical pathway allowed *E. coli* to proliferate in the blood. When this strain was fed to the leech in heat-inactivated blood, the *E. coli* strain was able to proliferate and establish itself in the gut of the leech. These data clearly show a heat-sensitive property of vertebrate blood remains active inside the leech and contributes to the unusual specificity of the leech digestive-tract symbiosis. Even at the strain level, Aeromonads have been shown to have varying degrees of resistance to complement (Janda, 1994), which could contribute to certain species or strains having a competitive advantage over others in their ability to colonize the leech. By feeding on vertebrate blood, medicinal leeches temporarily gain access not just to the nutritional value of the ingested blood meal but also acquire an immunological protection. We term this "procured" immunity to differentiate from the factors encoded by the leech genome.

### Innate immunity

#### *Antimicrobial peptides*

Antimicrobial peptides (AMPs) are an important part in the innate immune response of vertebrates, invertebrates, and plants. They are a diverse group of compounds that have the ability to kill a broad spectrum of bacteria. The majority of AMPs are cationic and amphiphilic and their most common mode of action is to disrupt the bacterial cell membrane (Ganz, 2003). As one might suspect they are most commonly found at the interface between the organism and environment (*e.g.*, skin and digestive tract) in order to prevent microbial invasion (Ganz, 2003).

AMPs have been identified in the gastrointestinal tract of vertebrates and invertebrates but to our knowledge, none have been discovered in the leech gut. However, an Expressed Sequence Tag library of the salivary transcriptome from *Macrobdella decora*, revealed a c-type lectin that could possess antimicrobial activity (Min *et al.*, 2010). The AMPs *Hm*-lumbricin and neuromacin, have been isolated in the *H. medicinalis* central nervous system and theromacin, theromyzin, and peptide B from the leech hemolymph of the distantly related *Theromyzon tessulatum* (Schikorski *et al.*, 2008; Tasiemski *et al.*, 2004; Tasiemski *et al.*, 2000). Theromacin and theromyzin were also detected in the leech intestinal epithelium, which makes it plausible that AMPs are present in the gut (Tasiemski *et al.*, 2004).

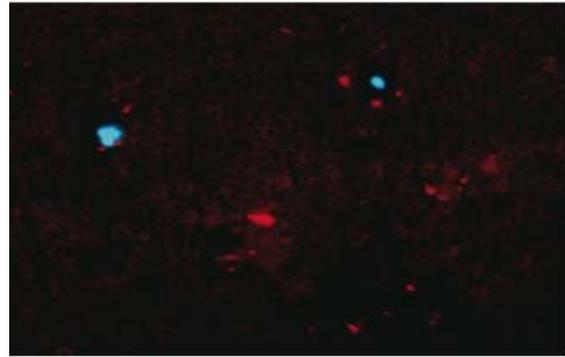
A mutation in the *A. veronii* Lpp, Braun's major outer membrane lipoprotein, led to a decreased ability for this strain to colonize the leech gut (Silver *et al.*, 2007b). Lpp is involved in membrane stability and subsequent resistance to toxic compounds such as antibiotics, cationic dyes, EDTA and the surfactants SDS and Triton X-100 (Hirota *et al.*,

1977; Sha *et al.*, 2004). Interestingly, some of these compounds share the core membrane disrupting characteristics as AMPs (*i.e.*, amphilic or cationic) (Ganz, 2003). The *A. veronii lpp* mutant exhibited an increased sensitivity to SDS but the weakened outer membrane was not affected by possible changes in osmolarity within the leech crop and was not sensitive to mammalian complement (Silver *et al.*, 2007b). These data suggested the observed colonization defect of the *lpp* mutant was possibly due to the presence of AMPs secreted into the leech gut (Silver *et al.*, 2007b). Besides membrane disruption and subsequent lysis, AMPs have also been shown to kill bacteria by binding intracellular targets (*e.g.* inhibiting DNA, RNA, protein, and cell-wall synthesis) (Brogden, 2005; Nicolas, 2009). AMPs gain intracellular access through mechanisms such as receptor-mediated endocytosis, membrane permeabilization, the formation of toroidal pores, and transient membrane leakage (Nicolas, 2009). Under this scenario a hypothetical AMP inside the leech gut would be able to traverse the membrane easier in the *lpp* mutant compared to wild-type *A. veronii*. This would lead to cell death and account for the colonization defect exhibited by this mutant, which provides indirect evidence for the presence of an AMP present inside the crop.

#### Phagocytic immune cells

Phagocytic immune cells represent another essential component of the innate immune response against invading pathogens. Immune cells referred to as amebocytes were first identified in the celomic fluid and connective tissue of *H. medicinalis* at which time it was proposed that they could play a role in phagocytosis (Sawyer, 1986). More recently, in the leech *Glossiphonia complanata*, macrophage-like cells, NK-like cells, and granulocytes were discovered in blood vessels, lacunae, and connective tissue (de Eguileor *et al.*, 2000b; de Eguileor *et al.*, 1999). When these leeches were stimulated via saline or LPS prick on their outer surface, these immune cells traveled from the area adjacent to the gut to the wound site (de Eguileor *et al.*, 2000b). de Eguileor *et al.* demonstrated the macrophage-like cells possess phagocytic activity (de Eguileor *et al.*, 2000a).

Utilizing fluorescence *in situ* hybridization (FISH), nucleated cells were identified in the gut of *H. verbana* after an ingested blood meal, however, it was possible these were sheep immune cells (Silver *et al.*, 2007a). The blood meal was reconstituted after several centrifugations and it was determined that over 95 % of the sheep derived leukocytes were removed before the leech was fed, thereby confirming these nucleated cells were of leech origin (Fig. 1) (Silver *et al.*, 2007a). In the same study an *A. veronii* type III secretion system (T3SS) mutant was shown to have a dramatically reduced ability to colonize the leech gut (Silver *et al.*, 2007a). The T3SS is a molecular needle complex that injects effector proteins into eukaryotic cells, which then elicit a multitude of host cell changes (Hueck, 1998). In other Gram-negative bacteria, many of these effectors contain N-terminal Rho GTPase-activating protein (RhoGAP) domains, which if expressed in eukaryotic cells disrupt the actin cytoskeleton and

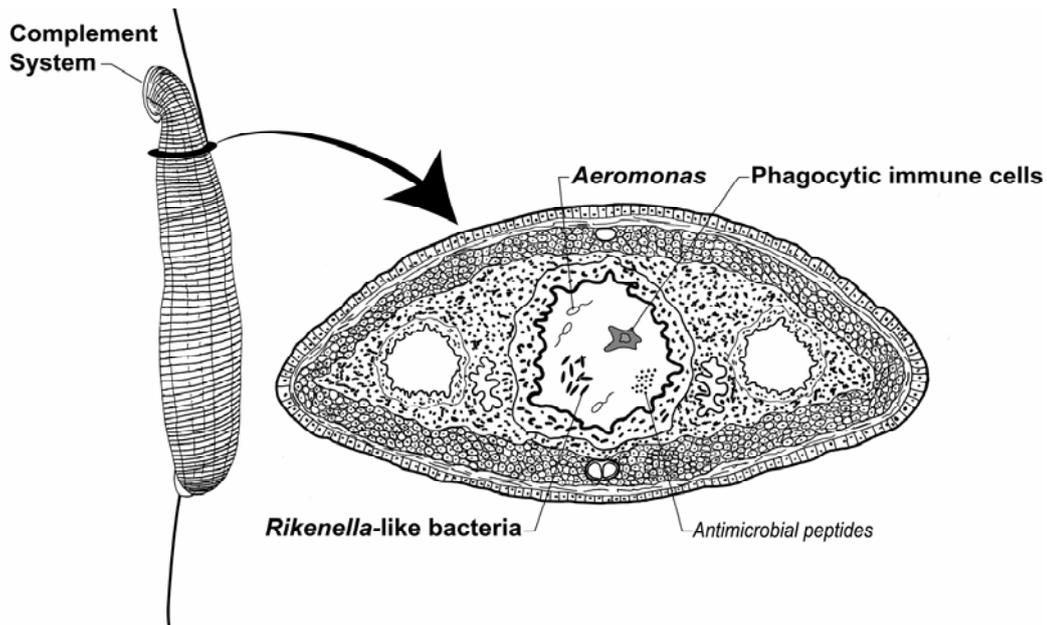


**Fig. 1** Fluorescence micrograph of *A. veronii* in the crop of the leech. Crop section of a leech inoculated with *Aeromonas* 42 h after feeding. *Aeromonas* (red) and nuclei (blue) were visualized by staining with an *Aeromonas*-specific probe and DAPI, respectively. Bacteria were either not associated with leech immune cells, or associated with immune cells via surface-association or intracellular association. Erythrocytes have a dark center surrounded by a red autofluorescence. Copyright 2007 National Academy of Sciences, USA

interfere with phagocytosis (Barbieri and Sun, 2004). After the T3SS mutant or wild-type strain were fed to the leech, fluorescence micrographs of the gut revealed the leech immune cells phagocytosed the mutant at a much higher frequency than wild-type *A. veronii* (Silver *et al.*, 2007a). This was evidenced by the mutant appearing tightly associated with the nucleus of the immune cell (*i.e.*, intracellularly located), while the wild-type strain remained on the outside of the cell, thereby resisting engulfment by use of its functional T3SS (Fig. 1). These data demonstrated that phagocytic immune cells infiltrate the leech digestive tract and remove susceptible bacteria and that *A. veronii* overcomes this barrier of the innate immune response through the use of a functional T3SS (Silver *et al.*, 2007a).

Another important aspect of the interaction of *A. veronii* and the leech immune cells is that *A. veronii* does not interfere with the phagocytosis of other cells. When the wild-type strain and the T3SS mutant were introduced together, the T3SS mutant still decreased dramatically in abundance, while the other strain proliferated (Silver *et al.*, 2007a). The lack of providing general protection may be an important aspect contributing to the specificity of the microbial community inside the digestive tract of *H. verbana*.

The ability of several *A. veronii* isolates to colonize the digestive tract of *H. verbana* was examined by competing each against a native *A. veronii* isolate from *H. verbana* in a standard competition assay, using heat-inactivated blood, in which the test and control strains carry different antibiotic resistance markers (Silver *et al.*, 2011). Strains isolated from another leech species, *H. orientalis* an eel, and several clinical isolates were



**Fig. 2** Cross section of the medicinal leech depicting procured (complement) and innate (immune cells and AMPs) immunity. The digestive-tract symbionts, *Aeromonas* and the *Rikenella*-like bacteria, are shown. Antimicrobial peptides are written in italics instead of bold to distinguish speculation versus evidence, as with complement and immune cells.

competed against the control strain. It was determined that the strain isolated from *H. orientalis* colonized to comparable levels inside the crop of *H. verbana* as the control strain (Silver *et al.*, 2011). However, all of the remaining isolates had a significantly reduced ability to colonize the leech, which ranged from a less than 10-fold defect to a greater than 4,000-fold defect (Silver *et al.*, 2011). Control experiments revealed that the colonization defect exhibited by the isolates was not due to an inability to proliferate in blood (Silver *et al.*, 2011). The inability of other closely related *A. veronii* isolates to colonize the leech crop further demonstrates the specificity of this microbial association.

### Microbial derived factors

It remains likely that other factors in addition to the complement of the ingested mammalian blood, AMPs, and immune cells can contribute to the innate immunity in the leech digestive tract. The symbionts within the leech gut, *A. veronii* and the *Rikenella*-like bacterium, could play an important role in supplementing the innate immune response within the crop. As proposed in other systems, the symbionts could prevent the colonization of pathogenic bacteria by occupying a specific niche or out competing pathogenic bacteria for nutrients. In addition, secreting AMPs, bacitracin, or anti-quorum sensing compounds could also shape the microbial community. The presence of a more diverse microbial population, which includes both *A. veronii*

and the *Rikenella*-like bacterium, in the intestine suggests they are not secreting AMPs or if they are, their expression is downregulated in the intestine. If the gut symbionts are not directly secreting AMPs they could be responsible for stimulating the gut epithelium to express increased levels of AMPs.

The digestive tract of *H. verbana* represents an interesting evolutionary adaptation where the immune system of an annelid is supplemented by components of the immune system from a vertebrate on which it feeds (Fig. 2). It is possible that the complement system of the ingested blood also remains active in the gut of other blood feeding animals. However, since many of these animals have not been amendable to the introduction of genetically manipulated symbionts during the feeding process, the series of factors that contribute to their specificity have not been revealed. In other model systems, such as the Hawaiian bobtailed squid, *Euprymna scolopes*, it is likely a series of factors contribute to the establishment of the normal microbiome (Nyholm and McFall-Ngai, 2004; Ruby, 2008) and inside *Hirudo verbana* multiple factors such as hemocytes and ingested complement contribute to this specificity (Silver *et al.*, 2007a; Braschler *et al.*, 2003).

### Acknowledgements

We would like to thank V Kask for the artwork. The research was supported by NSF Career Award MCB 0448052 and NIH R01 GM095390 to JG.

## References

- Barbieri JT, Sun, J. *Pseudomonas aeruginosa* ExoS and ExoT. Rev. Physiol. Biochem. Pharmacol. 152: 79-92, 2004.
- Braschler TR, Merino S, Tomas JM, Graf J. Complement resistance is essential for colonization of the digestive tract of *Hirudo medicinalis* by *Aeromonas* strains. Appl. Environ. Microbiol. 69:4268-4271, 2003.
- Broderick NA, Raffa, KF, Goodman, RM, Handelsman, J. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl. Environ. Microbiol. 70: 293-300, 2004.
- Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3: 238-250, 2005.
- de Eguileor M, Grimaldi A, Tettamanti G, Valvassori R, Cooper EL, Lanzavecchia G. Different types of response to foreign antigens by leech leukocytes. Tissue Cell 32: 40-48, 2000a.
- de Eguileor M, Grimaldi A, Tettamanti G, Valvassori R, Cooper EL, Lanzavecchia G. Lipopolysaccharide-dependent induction of leech leukocytes that cross-react with vertebrate cellular differentiation markers. Tissue Cell 32: 437-445, 2000b.
- de Eguileor M, Tettamanti G, Grimaldi A, Boselli A, Scari G, Valvassori R, et al. Histopathological changes after induced injury in leeches. J. Invertebr. Pathol. 74: 14-28, 1999.
- Ganz T. The role of antimicrobial peptides in innate immunity. Integr. Comp. Biol. 43: 300-304, 2003.
- Graf J. Symbiosis of *Aeromonas veronii* biovar *sobria* and *Hirudo medicinalis*, the medicinal leech: a novel model for digestive tract associations. Infect. Immun. 67: 1-7, 1999.
- Graf J. The symbiosis of *Aeromonas* and *Hirudo medicinalis*, the medicinal leech. ASM News 66: 147-153, 2000.
- Hirota Y, Suzuki H, Nishimura Y, Yasuda S. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. USA 74: 1417-1420, 1977.
- Hueck CJ. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62: 379-433, 1998.
- Indergand S, Graf J. Ingested blood contributes to the specificity of the symbiosis of *Aeromonas veronii* biovar *sobria* and *Hirudo medicinalis*, the medicinal leech. Appl. Environ. Microbiol. 66: 4735-4741, 2000.
- Janda JM, Guthertz LS, Kakka RP, Shimada T. *Aeromonas* species in septicemia: laboratory characteristics and clinical observations. Clin. Infect. Dis. 19: 77-83, 1994.
- Jung T. Zur Kenntnis der Ernährungsbiologie der in dem Raum zwischen Harz und Heide vorkommenden Hirudineen. Zool. Jb. 66: 79-123, 1955.
- Kikuchi Y, Graf J. Spatial and temporal population dynamics of a naturally-occurring, two-species microbial community inside the digestive-tract of the medicinal leech. Appl. Environ. Microbiol. 73:1984-1981, 2007.
- Maltz M, Graf J. The type II secretion system is essential for erythrocyte lysis and gut colonization by the leech digestive tract symbiont *Aeromonas veronii*. Appl. Environ. Microbiol. 77: 597-603, 2011.
- Min GS, Sarkar IN, Siddall ME. Salivary transcriptome of the North American medicinal leech, *Macrobdella decora*. J. Parasitol. 96: 1211-1221, 2010.
- Nicolas P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. FEBS J. 276: 6483-6496, 2009.
- Nyholm SV, McFall-Ngai MJ. The winnowing: establishing the squid-vibrio symbiosis. Nat. Rev. Microbiol. 2: 632-642, 2004.
- Rados C. Beyond bloodletting: FDA gives leeches a medical makeover. FDA Consum. 38: 9, 2004.
- Rigbi M, Levy H, Iraqi F, Teitelbaum M, Orevi M, Alajoutsijarvi, A., et al. The saliva of the medicinal leech *Hirudo medicinalis*-I. Biochemical characterization of the high molecular weight fraction. Comp. Biochem. Physiol. 87: 567-573, 1987.
- Rigbi M, Orevi M, Eldor A. Platelet aggregation and coagulation inhibitors in leech saliva and their roles in leech therapy. Semin. Thromb. Hemost. 22: 273-278, 1996.
- Rio RV, Maltz M, McCormick B, Reiss A, Graf J. Symbiont succession during embryonic development of the European medicinal leech, *Hirudo verbana*. Appl. Environ. Microbiol. 75: 6890-6895, 2009.
- Ruby EG. Symbiotic conversations are revealed under genetic interrogation. Nat. Rev. Microbiol. 6: 752-762, 2008.
- Sawyer RT. Leech biology and behavior. Clarendon Press, Oxford, UK, 1986.
- Schikorski D, Cuvillier-Hot V, Leippe M, Boidin-Wichlacz C, Slomianny C, Macagno E, et al. Microbial challenge promotes the regenerative process of the injured central nervous system of the medicinal leech by inducing the synthesis of antimicrobial peptides in neurons and microglia. J. Immunol. 181: 1083-1095, 2008.
- Sha J, Fadl AA, Klimpel GR, Niesel DW, Popov, VL, Chopra AK. The two murein lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. Infect. Immun. 72: 3987-4003, 2004.
- Siddall ME, Trontelj P, Utevsky SY, Nkamany M, Macdonald KS. Diverse molecular data demonstrate that commercially available medicinal leeches are not *Hirudo medicinalis*. Proc. Roy. Soc. Lond. 274: 1481-1487, 2007.
- Silver AC, Kikuchi Y, Fadl AA, Sha J, Chopra AK, Graf J. Interaction between innate immune cells and a bacterial type III secretion system in mutualistic and pathogenic associations. Proc. Nat. Acad. Sci. USA 104: 9481-9486, 2007a.
- Silver AC, Rabinowitz NM, Kueffer S, Graf J. Identification of *Aeromonas veronii* genes required for colonization of the medicinal leech, *Hirudo verbana*. J. Bacteriol. 189: 6763-6772, 2007b.

- Silver AC, Williams D, Faucher J, Horneman AJ, Gogarten JP, Graf J. Complex evolutionary history of the *Aeromonas veronii* group revealed by host interaction and DNA sequence data. *PLoS one*. 6: e16751, 2011.
- Tasiemski A, Vandenbulcke F, Mitta G, Lemoine J, Lefebvre C, Sautiere PE *et al.* Molecular characterization of two novel antibacterial peptides inducible upon bacterial challenge in an annelid, the leech *Theromyzon tessulatum*. *J. Biol. Chem.* 279: 30973-30982, 2004.
- Tasiemski A, Verger-Bocquet M, Cadet M, Goumon Y, Metz-Boutigue MH, Aunis D *et al.* Proenkephalin A-derived peptides in invertebrate innate immune processes. *Brain Res.* 76: 237-252, 2000.
- Whitaker IS, Cheung CK, Chahal CA, Karoo RO, Gulati A, Foo IT. By what mechanism do leeches help to salvage ischaemic tissues? A review. *Br. J. Oral Maxillofac. Surg.* 43: 155-160, 2005.
- Whitaker IS, Josty IC, Hawkins S, Azzopardi E, Naderi N, Graf J, *et al.* Medicinal leeches and the microsurgeon: a four-year study, clinical series and risk benefit review. *Microsurgery* 31: 281-287, 2011.
- Whitaker IS, Kanya C, Azzopardi EA, Graf J, Kon M, Lineaweaver WC. Preventing infective complications following leech therapy: is practice keeping pace with current research? *Microsurgery* 29: 619-625, 2009.
- Worthen PL, Gode CJ, Graf J. Culture-independent characterization of the digestive-tract microbiota of the medicinal leech reveals a tripartite symbiosis. *Appl. Environ. Microbiol.* 72: 4775-4781, 2006.
- Zebe E, Roters FJ, Kaiping B. Metabolic changes in the medical leech *Hirudo medicinalis* following feeding. *Comp. Biochem. Physiol.* 84A: 49-55, 1986.