Chapter - 2

Review of Literature
Severe burns remain a significant cause of morbidity and mortality despite the availability of numerous therapies (Martineau and Shek, 2006). Burns remain a huge public health issue at least in terms of morbidity and long-term disability throughout the world especially in developing countries where the risk of infection in severe burns is well known (Heimbach, 1999; Komolafe et al., 2003; Nasser et al., 2003). Burns have long been recognized among the most painful and devastating injuries a person can sustain and survive (Shakespeare, 2001; Church et al., 2006). Even in developing countries, more than 2 million individuals annually are burned seriously and require medical treatment (Cakir and Yegen, 2004). Burns can be thermal (from extremes of heat or cold), chemical (such as acid), or radiant (sunburn, X-rays or artificial ultraviolet rays from a tanning booth). All the mechanisms cause skin damage and this should be treated similarly. Burns due to hot objects (thermal burn) are the most common type (Groohi et al., 2002). Thermal and related injuries are a major cause of death and disability, especially in subjects under the age of 40.

Thermal injury destroys the skin barrier that normally prevents invasion by microorganisms, making the burn wound the most frequent origin of sepsis in these patients (Vindenes and Bjerknes, 1995; Nasser et al., 2003; McVay et al., 2007). Direct contact with flame, a hot surface or hot liquid (scald), or a source of heat conduction or convection, causes a degree of cellular damage to the skin that varies with the temperature and duration of exposure (Haberal et al., 1989; Peate, 1992; Rai et al., 1999; Koumbourlis, 2002). As the temperature rises, increased molecular collisions occur, resulting in altered molecular conformation and disruption of intermolecular bonds. This process leads to cell membrane dysfunction as ion channels are disrupted, resulting in sodium and water intake (Baker et al., 1980). As the temperature rises further, protein denaturation occurs, oxygen radicals are liberated, and eventually cells die with the formation of the burn eschar (Mayhall, 2003; Church et al., 2006). In addition, the thickness of the skin layer is critical as the thinner the skin, deeper the burn (McManus et al., 1987).
2.2 THE CLASSIFICATION OF BURNS

Burns are often described in terms of depth of injury. The depth of dermis lost is due to the degree of temperature of the burn and duration of exposure to the offending source. Burns are classified as first-, second-, or third-degree (Fig. 2.2), depending on how deep and severe they penetrate the skin's surface (Engrav et al., 1983; Soroff and Singer, 2005) but sometimes this is extended to include a fourth or even up to a sixth degree, but most burns are first to third-degree.

First degree
• Involves top layer of epidermis only

Second degree burn
• Skin blister
• Involves all of epidermis and some of dermis
• May involve all of the dermis

Third degree burn
• May extend into deeper tissues

Fig. 2.2. Burn classification
predisposes patients to subsequent sepsis and multiple organ failure, which are the major causes of morbidity and mortality in burn patients (Beal and Cerra, 1994; Harris and Gelfand, 1995; Baue et al., 1998; Schwacha and Chaudry, 2002).

2.4 IMMUNE RESPONSE TO BURN INJURY

Host defense against infection can be divided into innate and adaptive immune responses (Sparkes, 1997; Church et al., 2006). The innate immune response acts immediately after the integument system is breached and relies on a phylogenetically ancient system for microbial recognition in which germ line-encoded receptors recognize structural components of microorganisms (Steinstrasser et al., 2004). The adaptive immune response often takes longer, especially if it involves exposure to new antigens. Thermal injury may trigger a characteristic sequence of inflammatory events (Allgower et al., 1995) leading to major changes in immune cell populations, alteration in cell subset ratios, abnormal production of cellular immune factors, release of a variety of inflammatory mediators and alterations in cellular proliferation and protein synthesis, eventually resulting in an increased susceptibility to infection and risk of death (Deitch, 1992; Mathieu et al., 1994; O'Sullivan et al., 1995; O'Sullivan and O'Connor, 1997). Major burn injury leads to a profound depression of both humoral and cell-mediated immunity. A growing body of evidence suggests that the release of large quantities of pro-inflammatory cascade such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), or tumor necrosis factor (TNF) (Butler et al., 1992; Fassbender et al., 1993; de Bandt et al., 1994) after burn injury is responsible for the development of immune dysfunction, susceptibility to sepsis and multiple organ failure (Meakin, 1990; Schwacha and Chaudry, 2002; Church et al., 2006). Anti-inflammatory cytokines, such as interleukin-2 (IL-2), interleukin-4 (IL-4), or interleukin-10 (IL-10), are then released in order to balance the effects of the pro-inflammatory cytokines (Despond et al., 2001). Perturbations of pro- and anti-inflammatory cytokine expression results in altered immune function and protein metabolism, potentially leading to compromised
predisposes patients to subsequent sepsis and multiple organ failure, which are the major causes of morbidity and mortality in burn patients (Beal and Cerra, 1994; Harris and Gelfand, 1995; Baue et al., 1998; Schwacha and Chaudry, 2002).

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structure or function of the immune system, liver, skin, or muscle (Vindenes and Bjerknes, 1997; Wray et al., 2002; Schwacha, 2003). In addition, hypermetabolism leads to futile protein utilization resulting in induction of a dynamic hypercatabolic state concurrent with altered cytokine expression (Wray et al., 2002; Finnerty et al., 2006).

2.5 PATHOGENESIS OF BURN WOUND INFECTIONS

Serious thermal injury causes total loss of the skin surface over large areas of the body (Church et al., 2006). Because of the importance of the skin as a barrier to microbial host invasion, it is not surprising that the risk of subsequent burn wound infection and systemic infection correlates with the size of the burn injury (Sheridan, 2000; Santaniello et al., 2004). Thermal destruction of the skin barrier and concomitant depression of local and systemic host cellular and humoral immune responses are pivotal factors contributing to infectious complications in patients with severe burns (Heideman and Bengtsson, 1992; Lederer et al., 1999; Kumar et al., 1999; Cook, 1998; Landsdown, 2002). The burn wound is a dynamic living environment that will alter depending on both intrinsic factors (such as release of inflammatory mediators, bacterial proliferation) and extrinsic factors (such as dehydration, systemic hypotension, cooling) (Papini, 2004). The burn wound surface (in deep partial-thickness and in all full-thickness burns) is a protein-rich environment consisting of avascular necrotic tissue (eschar) that provides a favorable niche for microbial colonization and proliferation (Manson et al., 1992; Barret and Herndon, 2003; Nasser et al., 2003; Erol et al., 2004). The susceptibility of the burn wound to infection results from the combined effect of the presence of coagulated proteins and other microbial nutrients in the wound and the avascularity of the eschar, which prevents delivery of immunologically active cells, humoral factors, and even bloodborne antibiotics to the eschar (Hansbrough, 1987; Pruitt et al., 1998).

Burn wounds are susceptible to infection not only by common human pathogens, but also by organisms not normally pathogenic in the uncompromised host due to impairment of the skin barrier and reduction in cell mediated
immunity (Barlow, 1994; O’Sullivan and O’Connor, 1997; Miller, 1998; Landsdown, 2002). Although burn wound surfaces are sterile immediately following thermal injury, these wounds eventually become colonized with microorganisms (Wysocki, 2002). The microbiology of most wound types is complex, involving both aerobic and anaerobic bacteria (Bowler et al., 2001), and these organisms can create a potential problem to both the wound in which they reside (i.e. autoinfection) and the surrounding environment (cross-contamination) (O’Neill et al., 2003). The nature and extent of the thermal injury along with the types and amounts of microorganisms colonizing the burn wound appear to influence the future risk of an invasive wound infection (Manson et al., 1992; Barret and Herndon, 2003; Nasser et al. 2003). Gram-positive bacteria that survive the thermal insult, such as Staphylococci located deep within sweat glands and hair follicles, heavily colonize the wound surface within the first 48 hours unless topical antimicrobial agents are used (Gibson and Thompson, 1955; Altoparlak et al., 2004). Eventually (after an average of 5 to 7 days), these wounds are subsequently colonized with other microbes, including gram-positive bacteria, gram-negative bacteria, and yeasts derived from the host’s normal gastrointestinal and upper respiratory flora and/or from the hospital environment or that are transferred via a healthcare worker’s hands (Manson et al., 1992; Wurtz et al., 1995; Weber et al., 1997; Ramzy et al., 1998; Weber and McManus, 2004). Over the last several decades, gram-negative organisms have emerged as the most common etiologic agents of invasive infection by virtue of their large repertoire of virulence factors and antimicrobial resistance traits (Clark et al., 2003; Dalamaga et al., 2003). The principal portal of entry for pathogens in patients following thermal injury is the respiratory tract, with pneumonia accounting for 50% of burn-related deaths (Shirani et al., 1987). Burn wounds provide a suitable site for bacterial colonization. This colonization can lead to local invasive infection and systemic sepsis (Nagoba et al., 1998). Following colonization the organisms on the surface start to penetrate the burn eschar to a variable extent, depending on their invasive capacity, local wound factors, and the degree of patient’s immunosuppression (Nasser et al., 2003). Infection or
sepsis is present in a burn wound when deposition and multiplication of bacteria in the tissue is associated with a host reaction or invasion of nearby healthy tissue and there is a bacterial count of $10^5$ gm of tissue (Fong et al., 2005).

In a 5 year study reported from Govt. Medical College Hospital, Chandigarh, India, (June 1997–May 2002), *Pseudomonas aeruginosa* was found to be most common isolate (59%) followed by *Staphylococcus aureus* (17.9%), *Acinetobacter* spp. (7.2%), *Klebsiella* spp. (3.9%), *Enterobacter* spp. (3.9%), *Proteus* spp. (3.3%) and others (4.8%) (Agnihotri et al., 2004). During the period from 2002 to 2007, *Pseudomonas* species was still the commonest pathogen isolated (51.5%) among other burn wound bacterial isolates (Ahmad, 2002; Kaushik et al., 2003; Nasser et al., 2003; Singh et al., 2003). *Klebsiella* remains one of the most important gram negative isolates from burn wound associated infections. A retrospective study of bacterial infection in 71 burned patients over a 5-year period (1993-1997) was carried out in the burn unit at the National Hospital for Orthopaedic and Plastic Surgery, Enugu, Nigeria, of the 90 isolates, the most common organisms was *Klebsiella* spp. (26.7%) closely followed by *Staphylococcus aureus* (25.6%) and *Pseudomonas aeruginosa* (15.6%) (Ozumba and Jiburum, 2000).

Although modern antimicrobial therapy has improved the outcome of serious burn injury, infections remain a major cause of morbidity and mortality in patients surviving the shock phase of thermal injury (Pruitt and McManus, 1984; Jones et al., 1990; Mayhall, 2003). As infection is one of the most common causes of death after thermal injury, limited information is available on the association between bacterial colonization of burn wounds and survival outcome of burn patients (McManus et al., 1985; Mason et al., 1986; Panjusthahin et al., 2001). It has been estimated that at least 75-80% of all deaths caused by burns are the result of infection (Donati et al., 1993; O'Sullivan and O'Connor, 1997; Nasser et al., 2003; Church et al., 2006), and untreatable infections have become a tragically frequent occurrence in patients infected with multidrug resistant bacterial strains (McVay et al., 2007). Burn and effective burn therapy
have been considered as one of the major public health problems in the world (Rastegar and Alaghehbandan, 1999; Rastegar and Alaghehbandan, 2000; Rastegar et al., 2000; Alaghehbandan et al., 2001; Panjusthahin et al., 2001). Patients with serious thermal injury require immediate specialized care in order to minimize morbidity and mortality. Age has long been recognized as a major determining factor in burn mortality and elderly burn patients constitute a unique subgroup that imposes tremendous therapeutic challenge (Cutillas et al., 1998; Ho et al., 2001).

2.6 BURN WOUND DRESSING

Man has dressed wounds since life began many millions of years ago. One of the first things is to protect the wound from the influence of external forces or agents. For this there was, and is to this day, only one means—the application of a dressing (Queen et al., 1987). Since this time many materials have been devised from the intention of dressing wounds. Third degree burns need to be excised within few days and skin grafted. Biological dressings in past 20 years have become established as temporary (allografts and xenografts) and permanent (autograft) wound coverings (Bromberg et al., 1965; Zaroff et al., 1966; Rappaport et al., 1970; Wood and Hale, 1972). Biological dressings are natural tissues, usually skin, consisting basically of collagen sheets containing elastin and lipid (Bartlett, 1981). Pankhurst and Pochkhanawala, (2002) summarized the ideal burn dressing as one that must protect the wound from physical damage and micro-organisms, be comfortable, compliant and durable, be non-toxic, non-adherent, and non-irritant, allow gaseous exchange, allow high humidity at the wound, be compatible with topical therapeutic agents, be able to allow maximum activity for the wound to heal without retarding or inhibiting any stage of the process (Gore and Akolekar, 2003). The use of expensive commercially produced dressing products is not affordable in developing countries like India where economic reality is a fact of life.

The use of amniotic membrane, banana leaf dressing, honey, papaya and boiled potato peel bandages (BPPB) have all been reported in the literature (Dattatreyal et al., 1991; Starley et al., 1999; Ganatra and Fayyaz, 2007; Branski
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et al., 2008) as a wound cover for burns. The potato peel, which always finds a place in the kitchen bins, has found its place in the shape of an effective dressing material for burn injuries. BPPB was developed by Keswani and co-workers (Keswani and Patil, 1985; Patil and Keswani, 1985; Keswani et al., 1990; Subrahmanyam, 1994). This dressing was better accepted by the patients as compared to the vaseline gauze dressing and the pain during dressing change was considerably reduced. The banana leaf dressing (BLD) was subsequently developed and optimized by Gore and co-worker (1996) in Mumbai's Lokmanya Tilak Municipal General (LTMG) Hospital. An open controlled study was carried out by these workers to compare banana leaf dressing and boiled potato peel bandage. Both the dressings were observed to have equal efficacy in protecting the wounds and aiding healing but BLD was turned to be 11 times cheaper than BPPB. The leaves of banana are large thus offering larger surface area and the surface is non-adherent, waxy and cool. It is also the cheapest dressing available today (Gore and Akolekar, 2003).

An optimal dressing for burns and full thickness skin wounds is still a subject of current research. It is now accepted that a moist skin wound is an important factor for fast healing, including cell migration and formation of new epithelial cells resulting in normal epithelial tissue (Winter, 1962). The optimum approach for maintaining a moist wound surface is the use of occlusive dressings. These dressings include polyurethane films, a variety of hydrocolloids and an increasing use of temporary skin substitutes such as Biobrane™ and Transcyte™ in the treatment of mid- to deep-dermal burn injury (Barret et al., 2000; Kumar et al., 2004). Occlusive dressings using non-adherent material are best suited for this purpose. Petroleum jelly impregnated gauze is a popularly used dressing for these wounds in our country (Gao et al., 1992). Vloemans and co-workers (2001) developed a carboxymethylcellulose based hydrofibre dressing, Aquacel®, and tested for the treatment of partial thickness burns. The workers found it to be a safe, suitable and easy to use material for treatment of partial thickness burns. Martineau and Shek (2006), designed a medicated bi-layer wound dressing, chloramphenicol and chlorhexidine-loaded DRDC
hydrogel dressings which was effective in delivering medications, such as an antimicrobial agent, to the wound bed for the treatment of *P. aeruginosa* and *S. epidermidis*.

2.7 TOPICAL ANTIMICROBIAL AGENTS IN WOUND CARE

Infections in burn patients continue to be the primary source of morbidity and mortality. Topical antimicrobial therapy remains the single most important component of wound care in hospitalized burn patients (Monafo and West, 1990). The term "topical agent" implies the use of an antimicrobial agent applied to the surface of the wound (Ward and Saffle, 1995). The topical therapy of burn wounds dates back to the beginning of civilization (Moyer *et al.*, 1965). A wound that appears to be regressing should be cultured, the infecting agent should be identified, and the wound should be treated with debridement and application of an appropriate topical agent and dressing. A diverse variety of substances have been advocated as effective topical burn treatments including various plants, gums, milks (goat milk, and milk from "a women who has given birth to a son"), tea leaves, roasted angle worms, oak bark pextract, honey, cork, bear fat, bran, ashes, vinegar, wine, calcium chloride soaks, red sandalwood, cold water, saline baths, lemon strips soaked in oily dressings, soot, spider webs, linseed oil mixed with lime water, picric acid, medicated paraffin, carbolic acid and cod liver oil (Moncrief, 1971). Many effective antibacterial substances are now available for topical application in the prophylaxis of sepsis in burns (Nair *et al.*, 1991). Some of these products seem to be making a return, and other alternatives are being investigated (Mollering, 1995).

2.7.1 Topical Herbal Therapies

Herbal preparations are only one component of alternative medicine, which encompasses a wide variety of approaches. A large number of herbal therapies and combinations of therapies presently exist for wound care.

2.7.1.1 Honey

Honey is a mixture of sugars prepared by the bees from the natural sugar solutions called nectar obtained from flowers. Honey has been used in burn
wound treatment as long as 2000 years (Gupta et al., 1992; Subrahmanyam et al., 2001; Mathew and Binning, 2002). It is an ancient remedy which has been re-discovered for the treatment of wounds (Zumla and Lulat, 1989; Molan, 1998; 2001, 2002). Many therapeutic properties have been attributed to honey including antibacterial activity and the ability to promote healing (Molan, 1999; Molan and Betts, 2000). The antibacterial property of honey was first recognized in 1892 by Van Ketel (Dustman, 1979; Bangroo et al., 2005). Evidence of antibacterial activity is extensive, with more than 70 microbial species reported to be susceptible (Molan, 1992). Research indicates that honey has functional properties in human health promotion which depend largely on the floral source of the honey. The high viscosity, acidic pH, high osmolarity, and nutrient content of honey contribute to the inhibition of bacterial growth and promote wound healing (Efem, 1991; Subrahmanyam, 1991; Cooper et al., 1999, 2000, 2002; Dunford et al., 2000; Cooper et al., 2002; Molan, 2006) but geographical location, floral origin, and post-harvesting treatment conditions may also be important. The major antibacterial properties are related to the level of hydrogen peroxide determined by relative levels of glucose oxidase and catalase (Weston, 2000). The fact that the antibacterial properties of honey are increased when diluted, was clearly observed and reported in 1919. The explanation for this apparent paradox came from the finding that honey contains an enzyme that produces hydrogen peroxide when diluted. This agent was referred to as 'inhibine' before its identification as hydrogen peroxide (White et al., 1963; Molan, 1992). Honey has been found to be useful in the treatment of burns by helping the rapid healing of wounds (Tovey, 2000; Subrahmanyam et al., 2001; Jalali et al., 2007; Puljak et al., 2009).

2.7.1.2 Aloe vera

Aloe vera (Aloe vera Linn, synonym: aloe vera barbadensis Mill.) (Tamil – Southakathalai, Hindi – Ghikanvar), is a cactus-like plant with green, dagger-shaped leaves that are fleshy, tapering, spiny, marginated and filled with a clear viscous gel (Choi and Chung, 2003). The name was derived from the Arabic
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'aloeeh' meaning 'bitter', because of the bitter liquid found in the leaves. It is also known as 'lily of the desert', the 'plant of immortality' and the 'medicine plant' with qualities to serve as alternate medicine (Noor et al., 2008).

Aloe vera gel has been used for various ailments since the Roman era or even long before. It is present in the arid regions of India, and is believed to be effective in treating stomach ailments, gastrointestinal problems, skin diseases, constipation, for radiation injury (Noor et al., 2008), for its anti-inflammatory effect (Davis and Maro, 1989), for wound healing and burns (Chithra et al., 1998; Pribitkin and Boger, 2001). Burn wound healing is one of major indications of aloe vera gel use in many countries (Marshall, 1990; Visuthikosol et al., 1995; Reynolds and Dweck, 1999). Aloe vera has been reported for years to be effective in treating various types of burns (Somboonwong et al., 2000; Maenthaisong et al., 2007) including those resulting from radiation therapy.

2.7.1.3 Eucalyptus (Eucalyptus spp.)

Antibacterial properties of Eucalyptus against Pseudomonas aeruginosa have been reported in human burn patients. There are also a few reports in the veterinary literature that support the use of Eucalyptus in wound care (Agrawal, 1997; Bhagwat et al., 2000; Bansod, 2003).

2.7.2 Chemicals

Antiseptics are frequently used to cleanse animal wounds. These are used to reduce bacterial contamination by inhibiting the growth of microorganisms, and antiseptics should be applied to intact skin and not used directly on wounds as topical agents (Brown and Zitelli, 1993). Antiseptics may increase the intensity and duration of inflammation and they have also been shown to be toxic to human keratinocytes (Tarnall et al., 1987) and fibroblast (Viljanto, 1980; Lineaweaver et al., 1985). They also retard epithelialization. Iodine solutions and iodophors are often used as antiseptics.
Hydrogen peroxide is very commonly used as an antiseptic on wounds; however, it has limited bactericidal effectiveness as it is toxic to fibroblasts and impairs the microcirculation of wounds (Tarnall et al., 1987).

With an excellent spectrum of activity, low toxicity, and ease of application with minimal pain, silver sulfadiazine is currently the most extensively used topical agent for burn care in the United States (Taddonio et al., 1990). Silver sulphadiazine (1%) is thought to act via inhibition of DNA replication and modifications of the cell membrane and cell wall (Fox, 1968). Silver sulfadiazine is effective against a wide range of flora, particularly gram-negative bacteria (e.g. E. coli, Enterobacter, Klebsiella species, P. aeruginosa) including gram positive bacteria (e.g. S. aureus) and Candida albicans (Monafo and Ayvazian, 1978; Monafo and West, 1990; de Gracia, 2001).

Cerium nitrate has antimicrobial activity in vitro and reverses post-burn cell-mediated immunosuppression. The addition of cerium nitrate to silver sulphadiazine probably gives superior antimicrobial activity against gram positive and gram-negative organisms and fungus (Fox et al., 1977).

Mafenide acetate 0.5% cream (mafenide), a methylated topical sulfonamide compound was introduced prior to silver sulfadiazine and was widely used for the treatment of burns (Barillo, 2002). Until silver sulfadiazine was marketed, mafenide was the most widely used topical agent for burns. This drug has a wide range of antibacterial activity against most gram-negative and gram positive pathogens particularly Pseudomonas aeruginosa, but has little activity against gram-positive aerobic bacteria such as S. aureus (McCauley et al., 1989; Cooper et al., 1991; Ward and Saffle, 1995; Church et al., 2006).

Silver nitrate (0.5%) solution provides bactericidal activity against a wide range of bacterial flora, but is probably more effective against gram-positive bacteria (e.g. S. aureus). Development of resistance to silver ion is distinctly uncommon (Moyer et al., 1965; Percival et al., 2005). Dilute solutions of silver nitrate had been used since the 19th century to treat infections and burns before
the introduction of silver sulphadiazine cream (Fox, 1968; Ip et al., 2006). Silver-coated dressings are used extensively for wound management, particularly in burn wounds (Ross, et al., 1993; Caruso et al., 2004; Jones et al., 2004; Heggers, et al., 2005).

Chlorhexidine phosphanilate (CHP), a new broad-spectrum antimicrobial agent, has been evaluated as a topical burn wound dressing in cream form, but preliminary clinical trials reported that it was painful upon application (Miller et al., 1990). 0.25% sodium hypochlorite (NaOCl) solution (dakin's solution) is considered as a general bactericidal, fungicidal, and viricidal agent. Its use at concentrations as low as 0.025% has demonstrated bactericidal effects which in turn aids wound healing (Heggers et al., 1991). The use of sodium hypochloride as a topical agent was abandoned because of its basic pH, which causes pain and has a low antimicrobial effect (Noronha and Almeida, 2000).

Iodine is a potent antimicrobial agent that is frequently used in the management of wounds (primarily as povidone iodine or cadexomer iodine). It acts by destroying microbial protein and DNA (Steen, 1993; Ward and Saffle, 1995). Nitrofurazone 0.2% compound demonstrates a broad spectrum antibacterial activity as it is effective against S. aureus, Enterobactor, and E. coli, but it is less effective against P. aeruginosa than silver sulfadiazine or mafenide acetate. It has no significant fungicidal activity (Ward and Saffle, 1995; Noronha and Almeida, 2000).

Acetic acid at 0.5% concentration is bactericidal to many gram-negative and gram-positive microorganisms but is especially effective against P. aeruginosa (Phillips et al., 1968). Solutions of 0.25% acetic acid have also been reported, but they appear to be less effective in reducing microorganisms on wounds. This weak acid penetrates the cell wall and disrupts the cell membrane to establish its bactericidal effects (Sloss et al., 1993).

2.7.3. Antibiotics

Topical antibiotics are most effective when applied within three hours after wounding (Farstvedt et al., 2004). However, if the wound is completely debrided,
thus creating a new wound, they can be applied within three hours of debridement and are considered effective.

Gentamicin (0.1%) is effective against gram-negative organisms such as *Enterobacter, Klebsiella, and P. aeruginosa* (Snelling *et al.*, 1971; Snelling *et al.*, 1978). The mechanism of action of this agent appears to be inhibition of protein synthesis and messenger ribonucleic acid translation (Noronha and Almeida, 2000). On the other hand, bacitracin is used in the prophylaxis of gram positive bacterial infections of open areas (Jacob and James, 2004). Triple Antibiotic Ointment (Bacitracin, Polymyxin B, and Neomycin) (TA) has a wide antimicrobial spectrum but is ineffective against *Pseudomonas aeruginosa* (Swain, 1987; Zaki *et al.*, 1994; Farstvedt *et al.*, 2004). The zinc component of bacitracin has been shown to stimulate epithelialization (increasing it by 25%), but can retard wound contraction. These antimicrobials are poorly absorbed, therefore, toxicity is rare. Norfloxacin is used because of its broad spectrum antimicrobial activity. Its silver salts were formulated in a topical cream base. However, they warrant further development as topical anti-infective agents for use in treating burn patients (Noronha and Almeida, 2000).

Many effective antibacterial substances are now available for topical application in the prophylaxis of sepsis in burns. However, they have a few practical disadvantages. These include necessity of bulky cotton dressings (silver nitrate) or messy painful application if the agent is cream based (silver sulfadiazine, mafenide acetate and providone iodine). In addition, complications such as metabolic acidosis (Asch *et al.*, 1970), disturbances of thyroid function (Balogh *et al.*, 1985) and tissue deposition of silver (Bader, 1966) have been reported after their use. However, these compounds are no longer used extensively because significant resistance has developed and/or they have been shown to be toxic or ineffective at controlling localized burn wound infections (Palmieri and Greenhalgh, 2002). Emerging antimicrobial resistance in burn wound bacterial pathogens represent a serious therapeutic challenge for
clinicians caring for burn patients (Elliott and Lambert, 1999; Murphy et al., 2003; Altoparlak et al., 2004; Erol et al., 2004).

2.8 BACTERIOPHAGE THERAPY- AN EMERGING APPROACH

For more than half a century, the human society has been relying primarily on antibiotics to treat infectious diseases caused by pathogenic bacteria. However, the emergence of bacterial resistance to antibiotics following widespread clinical, veterinary, and animal or agricultural usage has made antibiotics less and less effective (Teuber 2001; Heuer et al., 2006). These days scientists are now facing the threat of superbugs, i.e. pathogenic bacteria resistant to most or all available antibiotics. It was warned by the World Health Organization (WHO) that those multiple antibiotic-resistant pathogens would very likely bring the world back to the pre-antibiotic era. Although the spread of antibiotic resistance has long been known as a worldwide phenomenon, research seems to have reached a dead end (Lorch, 1999). During the last 30 years, no new classes of antibiotics have been found, even with the help of modern biotechnology such as genetic engineering. Pharmaceutical companies have mainly focused on the development of new products derived from the known classes of antibiotics (Carlton, 1999; Sulakvelidze et al., 2001) which is a cause of major concern. Equally worrying is the fact that new antibiotics are not being developed at a rate sufficient to replace those drugs that are becoming less useful (Brussow and Kutter, 2005; Hanlon, 2007). Thus, exploring alternative approaches to develop antibacterial products is also a worthwhile task, and re-examining the potential of promising older methods might be of value (Alisky, et al. 1998; Thacker, 2003). As the window of opportunity for new antibiotic is rapidly closing, renewed interest in the possibilities of bacteriophage therapy- the harnessing of a specific kind of viruses that attack only bacteria to kill pathogenic microorganisms is seen (Levin and Bull, 1996; Barrow and Soothill, 1997; Alisky et al., 1998). Phage therapy - the therapeutic application of bacteriophages to control bacterial infections - has recently sparked interest as a potential alternative or complement to more traditional antibiotic therapy (Pirisi, 2000; Das, 2001; Kysela and Turner, 2007).
2.9 CHARACTERISTICS OF BACTERIOPHAGES

Bacteriophages ('eaters of bacteria', often known simply as 'phages') are naturally occurring viruses that infect bacteria (Carlton, 1999; Mathur et al., 2003; Payne and Jansen, 2003). Their general nature is similar to other viruses in that they consist of a piece of genetic information (nucleic acid) and a protein coat (Fig. 2.3), some containing lipids in their coats or envelopes (Matsuzaki et al., 2005). Typical phages have hollow heads (where the phage DNA or RNA is stored) and tunnel tails, the tips of which have the ability to bind to specific molecules on the surface of their target bacteria.

![Diagram of a typical bacteriophage](image)

Fig. 2.3. Diagrammatic representation of a typical bacteriophage.

Bacteriophages are highly specific for bacterial species, and multiply at the expense of the cell, eventually reducing the number of viable bacterial cells (Carlton, 1999; Sulakvelidze and Morris, 2001). They are highly specific, with most bacteriophages infecting only a single species of bacteria (Weber-Dabrowska et al., 2001). In many cases, only specific strains are infected (Nakai...
and Park, 2002; Hanlon, 2007). Bacteriophages occupy all those habitats of the
world where bacteria thrive (Skurnik and Strauch, 2006). Phages are ubiquitous
in our world- in the ocean, soil, deep sea vent, the water we drink and food we
eat and play key roles in regulating the microbial balance in every ecosystem
where this has been explored (Tartera and Jofre, 1987; Ashelford et al. 2000;
Hendrix 2002; Goodridge et al., 2003; Gorski et al., 2009). They require a
sensitive host with a specific receptor in order to replicate in the host cells
(Cherwonogrodzky, 2005). Phages like all viruses are absolute parasite. They
are metabolically inert in their extracellular form and have no machinery for
generating energy for making proteins (Bille et al., 2005). They reproduce by
insinuating themselves into the metabolism of the host bacteria (Marza et al.,
2006). When no appropriate host is present, many phages can maintain their
ability to infect for decades, unless damaged by external agents (Waldor and
Friedman, 2005).

2.10 CLASSIFICATION OF PHAGES

Bacteriophages are the most abundant organisms on earth, and it is
estimated that for each microbial isolate at least 10 different phages can be
found (Hendrix, 2002; Pedulla et al., 2003). These phages are classified into 13
families according to their morphological characteristics, type of nucleic acid, and
presence or absence of envelope or lipid. Over 96% of the phages described in
literature to date belong to order Caudovirales (tailed phages) which are
composed of an icosahedral head and a tail (Mathews, 1982; Maniloff and
Ackermann 1998; Ackermann, 1999). All those tailed phages have double-
stranded DNA (ds DNA) as genome and are lytic phages that encode endolysins,
also named virolysins (Young et al., 2000, Bernhardt et al., 2002). These phages
are classified into three families according to the morphological features of their
tail: Myoviridae (contractile tail), Siphoviridae (long flexible noncontractile tail),
and Podoviridae (extremely short non contractile tail).

The rest of the phages, constituting only 4% of the total, are classified into
10 families. They are cubic, filamentous, or pleomorphic phages with ds DNA,
single-stranded DNA (ss DNA), double-stranded RNA (ds RNA), or single-stranded RNA (ssRNA) genome (Ackermann 2001) (Fig. 2.4). Phages are classified by morphotype and host genus. The first six basic phage types, named A to F, were defined in 1967 on the basis of gross morphology and nature of nucleic acid (Bradley, 1967). New types were added over the years and the three types of tailed phages (A, B, C) were further subdivided according to head shape (Ackermann and DuBow, 1987). This subdivision by head shape is useful for the electron microscopist, but has little taxonomical importance. Phages may presently be divided into 21 morphotypes (Ackermann and DuBow, 1987; Van Regenmortel et al., 2000).

Fig. 2.4. Schematic representation of major phage groups

Bacteriophages undergo two possible life cycles. These are lytic (or virulent) or lysogenic (temperate) (Inal, 2003). These lytic phages use the host bacterium as a factory for their own replication (Petty et al., 2006; Sandeep, 2006). They can only multiply by means of lytic cycle; adsorb to the surface of the bacterial cells, reproduce to produce numerous progeny and get released by lysing the cell wall at the end of growth cycle (Fig. 2.5). In the process, each
phage particle can produce approximately 200 daughter phages per lytic cycle which can infect other bacteria (Carlton, 1999; Mathur et al., 2003; Theil, 2004; Mattey and Spencer, 2008). Temperate phages in contrast have a choice of reproductive modes when they infect a new host cell (Livermore, 2004).

These phages start their life cycle when they adsorb to permissive host. After injecting their genome into the host cell, they produce a set of early proteins and a few copies of their genome. On this stage a decision "lysis versus lysogeny" is made. Usually in poor growth conditions of the host cell, a phage chooses lysogenic pathway, because the number of progeny it can produce in such cell is usually low. When lysogeny is chosen, the phage integrates its genetic material with the host cell. When induction occurs through damage of DNA, the phage switches to the lytic cycle which results in the release of new phage particles (Lenski, 1988; Marza et al., 2006; Hanlon, 2007).

Fig. 2.5. Lytic cycle of a bacteriophage

Chapter 2
Temperate phages are seldom used in phage therapy because they do not kill 100% of the infected bacteria, and in certain cases, they contain genes that render the bacterium more virulent (Kropinski, 2006). The lethality and specificity of phages for particular bacteria, the ability of phages to replicate within infected animal hosts, and the safety of phages make them efficacious antibacterial agents (Duchworth and Gulig, 2002).

2.11 HISTORY OF BACTERIA EATER

The idea of using phages to fight bacteria dates back to the pre-antibiotic era (Bradbury, 2004). The concept of phage therapy to treat bacterial infections was born with the discovery of the bacteriophage almost a century ago (Inal, 2003; Theil, 2004). The history of bacteriophage discovery has been the subject of lengthy debates, including a controversy over claims for priority (Sulakvelidze et al., 2001). As much as a hundred years ago in 1896, Ernest Hankin, a British bacteriologist, reported something in the waters of the Ganges and Jumna rivers in India and he suggested that an unidentified substance (which passed through fine porcelain filters and was heat labile) had marked antibacterial activity, was responsible for limiting the spread of cholera epidemics (Sulakvelidze et al., 2001). Two years later, the Russian bacteriologist, Nikolay Fyodorovich Gamaleya observed a similar phenomenon while working with *Bacillus subtilis*, and latter the observations of several other investigators were also thought to be related to the bacteriophage phenomenon (Samsygina and Boni, 1984). However, none of these investigators further explored their findings until Frederick Twort, a medically trained bacteriologist from England, reintroduced the subject almost 20 years after Hankin’s observation by reporting a similar phenomenon and advancing the hypothesis that it may have been due to, among other possibilities, a virus (Sulakvelidze et al., 2001). However, for various reasons including financial difficulties, Twort did not pursue this finding and it was another 2 years before bacteriophages were "officially" discovered by Felix d'Herelle, a French-Canadian microbiologist at the Institute Pasteur in Paris reported the same phenomenon (Carlton, 1999; Summers, 2001). Intensive studies on the therapeutic use of phages for treating infectious diseases were
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taken up in 1920. *Vibrio cholerae* was the first bacterium against which first bacteriophage was isolated and phage therapy was tried but the activity of phages was found to be much higher *in vitro* than *in vivo* (Brock, 1961; Mathur et al., 2003). Phage was quite promising in treating cholera in a clinical setting, but phage always showed more lytic activity in culture than *in vivo* (Alisky et al., 1998). In the period after his discovery, d’Herelle promoted the use of phages as therapeutic agents for the treatment of infectious diseases and test of his belief in the therapeutic utility of phage were carried out in the field with avian typhosis (*Salmonella gallinarum*) and in the laboratory with *Shigella dysenteriae* infection of rabbits (Summers, 2001).

The first report on the bacteriophage therapy came in 1921 by Bruynoghe and Maisin who used bacteriophages to treat *Staphylococcus* skin disease (Bruynoghe and Maisin, 1921, Sulakvelidze et al., 2001). But at that time, physicians faced several problems regarding phage studies. Poor understanding of heterogeneity and ecology of the phages and the bacteria involved, difficulty in selecting appropriate mixture of phages of high virulence against the target bacteria (Skurnik et al., 2007) and failure to correctly and appropriately characterize the phage were some reasons which hampered the progress of these studies (Hanlon, 2007). However, due to the wide spread success, development of broad spectrum antibiotics and some inconsistent therapeutic results with the bacteriophages, research on bacteriophage therapy lost its importance (Kutter and Sulakvelidze, 2005). Hence development of phage therapy was largely discontinued in the West in 1940s with the emergence of penicillin and other chemical antibiotics, but it continued to be utilized in Eastern Europe and Former Soviet Union and today many infections untreatable with antibiotics can be treated in clinics in Georgia (Europe) and Poland (Alisky et al., 1998; Weber-Dabrowska et al., 2000; Ho, 2001; Parfitt, 2005; Clark and March, 2006). Phage therapy remained mostly inactive until the early 1980 when increasing concerns over the antibiotic resistant bacteria prompted researchers to reconsider bacteriophage therapy as an alternative to antibacterial agents (Lopez et al., 2004).
2.12 EARLIER PHAGE THERAPY STUDIES IN INDIA

Early years of his phage work, d'Herelle roamed the world exploring potential applications of phage therapy. One of his most publicized early successes was his 1925 treatment of four bubonic plague patients in Alexandria, Egypt. In 1920, while working at the Pasteur Institute Branch in Saigon, d'Herelle had isolated anti-plague phage from rat feces in the village of Bac Lieu during a severe plague outbreak (Summers, 1999). He used this phage in the treatment of bubonic plague. The report received much publicity, and d'Herelle was soon invited to conduct a large scale “Bacteriophage Inquiry” in India, in collaboration with Lt. Col. J. Morison, the acting director of the Haffkine Institute in Bombay. The study was initially for the efficacy of the phage treatment in plague and cholera patients but it focused on cholera (Burnet, 1930).

In 1927, at the Cambell Hospital in Culcutta under the direction of d'Herelle, a study was conducted on 27 patients of cholera using phages in carefully controlled fashion. During the study, orogastric administration of phages dramatically prevented cholera-associated fatalities in the hospital; the fatality rate dropped from 27-30% to zero. Similarly good results were observed during the field trials: prophylactic and therapeutic administration of phages to 74 patients in the villages of Panjab region reduced the mortality rate to 8%, as compared to 63% among 124 patients not treated with phages (d' Herelle et al., 1928). The results were very encouraging. The “Bacteriophage Inquiry” continued under the direction of Igor Asheshov, a Russian bacteriologist. Soon after Asheshov’s appointment, anti-cholera phages were used in the first-of-its-kind large scale attempt to use phages prophylactically, by reducing or eliminating environmental contamination with \textit{V. cholerae}. The phages were repeatedly poured into wells from which drinking water was obtained during the temporary lodging of pilgrims. Subsequent studies were conducted in Patna Medical College Hospital, where phage administration resulted in virtually no patients dying from cholera during the study period (Summers, 1999). The field trials were continued under the direction of Morison in Asian Province. The trials primarily focused on Naogaon and Habiganj villages—two widely separated, but comparable villages in Assam province. Both villages were endemic for cholera.
and here also phage therapy was successful. In subsequent years (for over a decade), bacteriophage administration was one of the most popular prevention and treatment methods for cholera in India. In 1938, the Shillong Pasteur Institute produced 400,000 doses of phages for the Bihar region alone and the demand from other regions continued to rise (Pollitzer et al., 1959; Hausler, 2003). In 1944, the Cholera Advisory Committee of the Indian Research Fund emphasized that no single approach can be a "magic bullet" to deal with infectious diseases.

2.13 PHAGE STUDIES IN POLAND AND FORMER SOVIET UNION

Phage therapy has been very common in Poland and Former Soviet Union. Table 2.1 discusses some of the major phage therapy studies carried out in these countries during period 1960 - 2001.

Table 2.1: Major phage therapy studies carried out during period 1960-2001.

<table>
<thead>
<tr>
<th>Reference(s)</th>
<th>Etiologic agent(s)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Babalova et al., (1968)</td>
<td>Shigella</td>
<td>Phages were successfully used for prophylaxis of bacterial dysentery.</td>
</tr>
<tr>
<td>Sakandelidze and Meipariani (1974)</td>
<td>Staphylococcus, Streptococcus, and Proteus</td>
<td>Phages administered subcutaneously or through surgical drains in 236 patients having antibiotic-resistant bacteria eliminated the infections in 92% of the patients.</td>
</tr>
<tr>
<td>Zhukov-Verezhnikov et al., (1978)</td>
<td>Staphylococcus, Streptococcus, E. coli, and Proteus</td>
<td>The superiority of adapted phages over commercial phage preparations was reported in treating 60 patients having suppurative infections.</td>
</tr>
<tr>
<td>Ioseliani et al., (1980)</td>
<td>Staphylococcus, Streptococcus, E. coli, and Proteus</td>
<td>Phages were successfully used together with antibiotics to treat lung and pleural infections in 45 patients.</td>
</tr>
<tr>
<td>Tolkacheva et al., (1981)</td>
<td>E. coli and Proteus</td>
<td>Phages were used together with bifidobacteria to treat bacterial dysentery in 59 immunosuppressed leukemia patients. The superiority of treatment with phage-bifidobacteria over antibiotics was reported.</td>
</tr>
<tr>
<td>Authors</td>
<td>Species</td>
<td>Results</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Meladze et al., (1982)</td>
<td>Staphylococcus</td>
<td>Phages were used to treat 223 patients having lung and pleural infections, and the results were compared to 117 cases where antibiotics were used. Full recovery was observed in 82% of the patients in the phage-treated group, as opposed to 64% of the patients in the antibiotic-treated group.</td>
</tr>
<tr>
<td>Cislo et al., (1987)</td>
<td>Klebsiella, Proteus, and E. coli</td>
<td>Thirty one patients having chronically infected skin ulcers were treated orally and locally with phages.</td>
</tr>
<tr>
<td>Slopek et al., (1983, 1984, 1985, 1987)</td>
<td>Staphylococcus, Pseudomonas, E. coli, Klebsiella, and Salmonella</td>
<td>A total of 550 patients were treated with phages. The overall success rate of phage treatment was 92%.</td>
</tr>
<tr>
<td>Weber-Dabrowska et al. 1987)</td>
<td>Staphylococcus and various gram negative bacteria</td>
<td>Orally administered phages were used to successfully treat 56 patients, and the phages were found to reach the patients’ blood and urine.</td>
</tr>
<tr>
<td>Kochetkova et al., (1989)</td>
<td>Staphylococcus and Pseudomonas</td>
<td>A total of 131 cancer patients having post surgical wound infections participated in the study. Of these, 65 patients received phages and the rest received antibiotics. Phage treatment was successful in 82% of the cases, and antibiotic treatment was successful in 61% of the cases.</td>
</tr>
<tr>
<td>Sakandelidze (1991)</td>
<td>Staphylococcus, Streptococcus, E. coli, Proteus, Enterococci, and P. aeruginosa</td>
<td>A total of 1,380 patients having infectious allergoses were treated with phages (360 patients), antibiotics (404 patients), or a combination of phages and antibiotics (576 patients). Clinical improvement was observed in 86, 48 and 83% of the cases, respectively.</td>
</tr>
<tr>
<td>Reference</td>
<td>Organisms</td>
<td>Summary</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
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<tr>
<td>Bogovazova et al., (1992)</td>
<td><em>K. ozaenae</em>, <em>K. rhinoscleromatis</em>, and</td>
<td>Phages were tested for any adverse effect in laboratory animals. They</td>
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<tr>
<td></td>
<td><em>K. pneumoniae</em></td>
<td>were found to be non toxic and non allergic. Adapted phages were</td>
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<td></td>
<td></td>
<td>reported to be effective in treating <em>Klebsiella</em> infections in all of the</td>
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<tr>
<td></td>
<td></td>
<td>109 patients examined during the study.</td>
</tr>
<tr>
<td>Miliutina and Vorotyntseva</td>
<td><em>Shigella</em> and <em>Salmonella</em></td>
<td>The effectiveness of treating salmonellosis using phages and a</td>
</tr>
<tr>
<td>(1993)</td>
<td></td>
<td>combination of phages and antibiotics was examined. The combination</td>
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<td></td>
<td></td>
<td>of phages and antibiotics was reported to be effective in treating</td>
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<tr>
<td></td>
<td></td>
<td>cases where antibiotics alone were ineffective.</td>
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<tr>
<td>Kwarcinski et al., (1994)</td>
<td><em>E. coli</em></td>
<td>Recurrent subphrenic abscess (after stomach resection) caused by an</td>
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<tr>
<td></td>
<td></td>
<td>antibiotic-resistant strain of <em>E. coli</em> was successfully treated with</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phages.</td>
</tr>
<tr>
<td>Perepanova et al., (1995)</td>
<td><em>Staphylococcus</em>, <em>E. coli</em>, and <em>Proteus</em></td>
<td>Adapted phages were used to treat acute and chronic urogenital</td>
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<tr>
<td></td>
<td></td>
<td>inflammation in 46 patients. The efficacy of phage treatment was</td>
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<tr>
<td></td>
<td></td>
<td>92% (marked clinical improvements) and 84% (bacteriological clearance).</td>
</tr>
<tr>
<td>Stroj et al., (1999)</td>
<td><em>K. pneumoniae</em></td>
<td>Orally administered phages were used successfully to treat meningitis in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a newborn (after antibiotic therapy failed).</td>
</tr>
<tr>
<td>Lazareva et al., (2001)</td>
<td><em>Staphylococcus</em>, <em>Streptococcus</em> and</td>
<td>Fifty four patients out of ninety four patients were treated with</td>
</tr>
<tr>
<td></td>
<td><em>Proteus</em></td>
<td>bacteriophages. Phage treatment was associated with fewer septic</td>
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<td></td>
<td></td>
<td>complications and temperature normalization compared to no phage</td>
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<tr>
<td></td>
<td></td>
<td>treatment. Microbiologically phage treatment showed a 2-fold reduction</td>
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<td></td>
<td></td>
<td>in the number of <em>Staphylococci</em> and <em>Streptococci</em> and 1.5 fold</td>
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<tr>
<td></td>
<td></td>
<td>reduction in the number of <em>Proteus</em> recovered from the burn wounds.</td>
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</tbody>
</table>
2.14 MODE OF ACTION AND SPECIFICITY OF BACTERIOPHAGES

Bacteriophages (phages) are bacterial viruses that infect bacterial cells, disrupt bacterial metabolism and cause the bacterium to lyse (Payne et al., 2000; Gorski et al., 2003; Theil, 2004). Efficacy of phage therapy is due to the bacteriolytic action of phages which occurs naturally at the end of the phage lytic cycle, by disruption of the cell wall caused by the virolysin-holin system or the single lytic factor (Levin and Bull, 2004). It could also happen in the adsorption stage if a high multiplication of infection (MOI) is used, in which a substantially large number of phage particles attach to the same bacterial cell (Tarahovsky et al., 1994). Another mode of action involves genetically modified phages, especially filamentous ones, which do not cause cell lysis and cannot be used directly for phage therapy. Hagens and Blasi, (2003) and Hagens et al., (2004) have shown that P. aeruginosa filamentous phage can be genetically modified by replacing the transportation gene (i.e. the gene involved in the extrusion of phage particles from the host bacterium) with a restriction enzyme gene so that the phages loses the ability to extrude from bacterial cells for its multiplication, but acquires the ability to digest the bacterial nucleic acid. They have demonstrated that this type of genetically modified filamentous phages could be used as effective anti-infection agents and have the benefit to reduce the release of membrane-associated endotoxins (lipopolysaccharide), leading to significantly higher rates of survival of experimental mice in comparison with therapies using lytic phages (Parisien et al., 2008).

The specificity of phages comes from the first essential step of phage infection cycle: the attachment of phage tail to a specific receptor on the surface of the bacterial cell (Lorch, 1999). There seem to have been some controversies with regard to how specific a phage could really be. While some authors tend to think that phages are species-specific (Merril et al., 2003), it seems to be more accurate to say that phages typically attack bacteria on a strain-specific basis (Bradbury, 2004). Nevertheless, there is no doubt that phages are much more specific than antibiotics (Theil, 2004; Matsuzaki et al., 2005; Hanlon, 2007). While this high specificity of phages made them less appealing in comparison...
with broad-spectrum antibiotics in the early days, it is now considered as a major merit because phage therapy would not affect the microbial flora of the host like more broad-spectrum antibiotics (Sulakvelidze et al., 2001; Skurnik and Strauch, 2006).

2.15 NON-TOXICITY OF PHAGE PREPARATIONS

The phages are composed of protein and DNA, few allergic or toxic effects would be expected provided that highly purified phage preparations are used. No such effects were seen when phage was given intravenously to 200 people as a test of immune function (Wedgwood et al., 1975). Bacteriophages are, however, good immunogens and an immune response has been reported during some phage therapy trials (Clark and March, 2006). Early phage therapy products were filter sterilized lysates containing bacterial debris with potential endo- and exotoxic compounds derived from host toxins released into lysates. Attempts have been made in recent times to get rid of these problems. Efficient purification of the phage can be achieved by CsCl gradient centrifugation and also by ammonium sulphate precipitation (Merril et al., 2006). A method for preparation of endotoxin-free bacteriophages by ultrafiltration and two-step chromatography was published recently by a Polish group (Boratynski et al., 2004). Small amounts of endotoxins may not pose a huge problem, as the human gut seems to have relatively low sensitivity to endotoxins applied orally (Clark and March, 2006). Whilst allergic effects have been seen in experimental animals treated with un-purified phage consisting of crude bacterial lysates, there are no reports of allergy seen with purified preparations (Clark and March, 2006). Proteomics might be one way to identify proteins in phage particles that could potentially cause allergic symptoms (Eyer et al., 2006).

There are phages for which the storage material of genetic information is DNA where one of the normal nucleotides is chemically modified (Gommers-Ampt and Borst 1995; Warren 1980). The risk connected with these phages is that the enzymes needed for the synthesis or modification of their DNA may be mutagenic. For example, *Bacillus subtilis* phages PBS1 and PBS2, having
genome composed of DNA where cytosine is replaced by uracil (Takahashi and Marmur, 1963). The phage enzyme that inhibits the breakdown of U-DNA, uracil-DNA glycosylase, was found to increase mutation frequency when expressed in human cells (Radany et al., 2000). It is likely that such phages are far more common than this far thought. Thus, care should be taken to recognize phages having modified bases in their DNA (Skurnik et al., 2007).

2.16 PHAGE PRODUCTS AS EFFECTIVE ANTIBACTERIALS

Virolysins are bacterial cell wall hydrolases encoded by lytic dsDNA phages and produced in phage-infected bacterial cells towards the end of the phage lytic cycle (Fischetti, 2008). The name ‘virolysin’ was first adopted by Ralston in the 1950s (Ralston et al., 1955). However, virolysins are now most commonly referred as endolysins, and were sometimes called lysozymes, lysins, and lytic enzymes. Virolysins are capable of degrading peptidoglycan when applied (as purified proteins) on bacterial cells, resulting in rapid lysis of the bacteria. They possess several important features including a narrow antibacterial spectrum and activity against bacteria regardless of their antibiotic sensitivity (Borysowski et al., 2006). A number of groups have advocated the use of purified lysins as a therapeutic tool rather than infective phage (Loessner, 2005). They have advantages over antibiotics in that they possess the host specificity of phages and so do not adversely affect normal microflora; there is less opportunity for resistance to emerge and they kill colonising pathogens on mucosal surfaces (Fischetti, 2005). Their major disadvantage is that they are unable to penetrate the outer membrane of gram negative cells and so their therapeutic activity is almost entirely directed at gram-positive infections.

Nelson et al., (2001) purified the lysin from C1 bacteriophage lytic for the group C Streptococcus strain 26RP66 and then used it both to prevent and eliminate colonization of mice by group A Streptococci. They showed that oral administration of the lysin did not affect the indigenous microflora but was rapidly lethal to the group A streptococci colonizing the mucosal surface of the upper respiratory tract. The preparation was non-irritant and did not induce any
mucosal immune response owing to the small quantities applied and its rapid action. Increasing concerns over terrorist attacks using biological weapons has led to the exploration of alternative therapies for the most likely candidate agent, anthrax. Inhaled spores can germinate in the lymph nodes surrounding the lungs and secrete toxins into the blood, which is nearly always fatal in untreated patients. Schuch et al., (2002) isolated the PlyG lysin from a Bacillus anthracis bacteriophage and showed that this enzyme could kill vegetative cells and germinating spores of the anthrax bacterium both in vitro and in vivo. This approach has been demonstrated to be efficient and safe antimicrobials, and could potentially be used for the control of pathogens on mucous membranes or as biowarfare countermeasures for Bacillus anthracis (Schuch et al., 2002; Fischetti et al., 2006). This concept has been taken a step further by Gaeng et al., (2000) who cloned the endolysin genes ply118 and ply511 from bacteriophages of Listeria monocytogenes into Lactococcus lactis. Additional enzymes have been listed that are potentially useful for treatment of some mucosal and other infections in animals and humans, such as PlyV12 from an Enterococcus faecalis phage (Yoong et al., 2004) and Ply3626 from a Clostridium perfringens phage (Zimmer et al., 2002). Lysostaphin has successfully been used to treat S. aureus infections, and Staphylococal phage lysins certainly have potential for application (Loessner et al., 1998, 1999).

Interestingly, virolysins are rapidly effective at low dosages in the order of milligrams or even micrograms per litre (Fischetti, 2005; Fischetti et al., 2006). This rapid killing of sensitive bacteria by virolysins at relatively low dosage is not only important in the sense of therapy costs, but could be one way whereby the enzymes would avoid being neutralized by the immune response or causing severe allergic responses in hosts (Fischetti 2005; Parisien et al., 2008).

2.17 BENEFITS OF PHAGE THERAPY OVER ANTIBIOTICS

Lytic phages are similar to antibiotics in that they have remarkable antibacterial activity. However, therapeutic phages have some at least theoretical advantages over antibiotics and phages have been reported to be more effective
than antibiotics in treating certain infections in humans (Meladze et al. 1982; Kochetkova et al. 1989; Sakandelidze, 1991) and experimentally infected animals (Smith and Huggins, 1982). Matsuzaki et al. (2005) summarized the advantages of phage therapy over antibiotic therapy and these are presented in Table 2.2.

Table 2.2: Comparison of phages and antibiotics regarding their prophylactic and therapeutic use

<table>
<thead>
<tr>
<th>Bacteriophages</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production is simple and inexpensive</td>
<td>Production is expensive</td>
</tr>
<tr>
<td>Phages are highly effective in killing their targeted bacteria i.e., their action is bactericidal</td>
<td>Some antibiotics are bacteriostatic, i.e., they inhibit the growth of bacteria, rather than killing them (e.g., chloramphenicol).</td>
</tr>
<tr>
<td>The high selectivity of bacteriophages permits the targeting of specific pathogens, without affecting desirable bacterial flora which means that phages are unlikely to affect the colonization pressure&quot; of the patients</td>
<td>Antibiotics attack not only the disease causing bacteria, but also all susceptible microorganisms including the normal microflora of the host. Thus their non-selective action affects the patient's microbial balance, which may lead to various side effects.</td>
</tr>
<tr>
<td>Because of phages specificity, their use is not likely to select for phage resistance in other (non-target) bacterial species</td>
<td>The broad spectrum activity of antibiotics may select for resistant mutants of many pathogenic bacterial species.</td>
</tr>
<tr>
<td>Humans are exposed to phages throughout life, and well tolerate them. Only few minor side effects and they may have been reported for therapeutic phages, and they may</td>
<td>For antibiotics multiple side effects including intestinal disorders, allergies and secondary infections have been reported.</td>
</tr>
</tbody>
</table>

Chapter 2
have been due to the liberation of endotoxin from bacteria lysed *in vivo* by the phages

<table>
<thead>
<tr>
<th>Because of phages specificity, their successful use for preventing or treating bacterial infections requires identification of the etiologic agent and determining its <em>in vitro</em> susceptibility to phage prior to initiating phage treatment</th>
<th>Antibiotics have higher probability of being effective when administered before the identity of the etiologic agent is known</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selecting a new phage (e.g., against phage-resistant bacteria) is a rapid process and frequently can be accomplished in days.</td>
<td>Developing a new antibiotic (against antibiotic resistant bacteria) is a time consuming process and may take several years to accomplish</td>
</tr>
</tbody>
</table>

### 2.18 BACTERIOPHAGE RECEPTORS AND EMERGENCE OF PHAGE RESISTANCE

One of the initial steps is the binding of the viral particle to the outer membrane of a bacterium (Moldovan *et al.*, 2007). The bacteriophage infection process starts by the specific recognition between the phage receptor-binding proteins (RBP) located at the tip of the tail and the receptor distributed over the host cell surface (Dupont *et al.*, 2004). Bacterial receptors have been well studied in gram-negative bacteria, particularly in *Escherichia coli*, while similar information lags behind in gram-positive bacteria (Duplessis and Moineau, 2001). For this purpose phages can use bacterial capsules, cell wall, flagella, fimbriae, different parts of lipopolysaccharide (LPS), and many other surface proteins as receptors (Skurnik and Strauch, 2006). The cell wall of gram negative bacteria has a mosaic of phage specific receptors. It is well known that lipopolysaccharide (LPSs) and lipoproteins are the two major components of the outer layer of the cell wall of gram negative bacteria (Totsuka, 1988). Bacteriophages may also use enzymes to break down capsule-like materials on the bacterial surface in a
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drill-like manner to reach the cell wall of the bacterium. A good example of such a phage is PK1A that uses the endosialidase activity of its tail to degrade the polysialic acid capsule of *E. coli* K1 (Pelkonen *et al.*, 1992). By mutating or losing the phage receptor bacteria become resistant to phages in question. This may not always be bad since resistance may reduce the fitness of the bacteria or if the receptor used by the phage is a virulence determinant, loss of the receptor would decrease the virulence of the bacterium dramatically (Levin and Bull, 2004).

The emergence of phage resistant bacterial mutants was observed soon after the discovery of bacteriophages, and the phenomenon was suggested to be a potential problem of phage therapy (Summers, 1999). Bacterial resistance to phages will unquestionably develop, although according to some authors (Carlton, 1999; Sulakvelidze *et al.*, 2001) the rate of developing resistance to phages is approximately 10-fold lower than that to antibiotics. However, in real life, the emergence of phage resistant mutants was not noted to be a problem in studies reported from Poland and Former Soviet Union, and phage-resistant mutants have been observed in the few *in vivo* and *in vitro* studies where phage treatment surviving colonies were examined for susceptibility. Bull *et al.*, (2002) recently reported that number of phage-resistant mutants - by the end of the treatment is less than 20% of the overall host strain population. Also, Leverentz *et al.*, (2001) were not able to detect phage resistant *Salmonella* mutants while examining the value of using bacteriophages as a biocontrol method for *Salmonella* on fresh cut fruits. The frequencies of the spontaneous mutation that confer phage resistance have not been determined for various phages (Drake *et al.*, 1998). Also, the phage resistance rates could be further reduced by using mixture of phages i.e. phage cocktails. The phage cocktails usually contain several lytic phages active against the same bacterial strain and their combined use is similar to the rationale of using two or more known antibiotics which is known to reduce the emergence of antibiotic resistant bacterial mutants (Carlton, 1999).
2.19 SAFETY PROFILE OF PHAGES

From a clinical standpoint, phages appear to be very safe. This feature is not surprising, given that humans are exposed to phages from birth. Bacteriophages are the most ubiquitous organisms on earth; e.g., one milliliter of non polluted water has been reported (Bergh et al., 1989) to contain $2 \times 10^8$ PFU of phages per ml, and the total number of phages on earth has been estimated to be in the range of $10^{30}$-10$^{32}$ (Brussow and Hendrix, 2002; Hanlon, 2007). Phages are normally found in the gastrointestinal tract, skin, and mouth, where they are harbored in saliva and dental plaque (Yeung and Kozelsky 1997; Bachrach et al., 2003). Ojeniyi et al., (1991) have found phages in the sputum samples of all 16 cystic fibrosis patients; this suggests that the sputum may be another ecosystem where phages can exert their action. Finally, phages have been also isolated from the urine of a patient with urinary tract infection (Caroli et al., 1980). Phages are abundant in saltwater, freshwater, soil, plants and animals and they have been shown to be unintentional contaminants of some vaccines and sera commercially available in United States (Merril et al., 1972; Geier et al., 1975; Milch and Fornosi, 1975). Phages also occur in food, including such traditionally ‘probiotic’ products as yogurt and sauerkraut (Kilic et al., 1996; Gorski and Weber-Dabrowska, 2005). The abundance of phages in the environment and the continuous exposure of humans to them - explain the extremely good tolerance of the humans to phages.

Side effects can develop due to the therapeutic action of phages itself, as well as a result of using insufficiently purified phage preparations therapeutically. Some side effects during phage therapy may be triggered by the in vivo lytic activity of phages against the etiologic agent e.g., liver area pain in a patient under going phage therapy in Poland was suggested to be related to extensive liberation of endotoxin from the lysed disease causing bacteria (Slopek et al., 1987). Similar complications also may be observed during antibiotic therapy (Prins et al., 1994). This complication can be avoided in those types of infection where this reaction is likely to occur by using genetically engineered bacteriophages; which have had their gene responsible for producing endolysin...
removed. Without this gene the host bacterium still dies but remains intact because apoptosis is disabled. Eventually these dead cells are consumed by the normal house cleaning duties of the phagocytes, which utilise enzymes to break the whole bacterium and its contents down into its harmless sub-units of proteins, polysaccharides and lipids. Purified phages have been injected intravenously into HIV infected patients (Fogelman et al., 2000), patients with other immuno deficiency diseases (Ochs et al., 1971) and healthy volunteers (Ochs et al., 1993) – strongly suggested that phage therapy may provide one of the safest as well as most environment friendly methods currently available for prophylaxis and treatment of bacterial infections. In order to compromise the safe use of therapeutic phage preparations, rigorous characterization of each phage to be used therapeutically should be done; in particular screening of potentially harmful genes in their genomes (Carlton et al., 2005; Hanlon, 2007).

2.20 NON SPECIFIC MECHANISMS THAT CLEAR BACTERIOPHAGES FROM BLOOD STREAM

Phage persistence in the mammalian organism has been proposed to have an impact on the efficacy of phage treatment because rapid elimination of phages from the mammalian host might reduce the number of phages to a level which is not sufficient to combat the infecting bacteria (Merril et al., 1996). Early phage investigators conducted such studies where they would have discovered that bacteriophages (being foreign proteins) tend to be rapidly cleared from the blood circulation. Multiple mechanisms may potentially contribute to the disappearance of phages from the mammalian bloodstream. Antibody responses would be one such mechanism but may take several days to occur. The innate immune system (reticuloendothelial system or RES) is another possible mechanism that is likely to be primarily responsible for the removal of phages from the mammalian bloodstream shortly after their administration. This clearance problem was first documented by Geier and his colleagues in 1973 who injected high titers of phage lambda into non-immune germ-free mice. They discovered that the phages were rapidly cleared by the spleen, liver and other filtering organs of the RES (Carlton, 1999). To address this issue, Merril et al.,
(1996) used a serial passage technique in mice to obtain a phage mutant capable of evading the reticuloendothelial system and therefore capable of long circulation in the blood due to minor variations in their coat proteins which enable some variants to be less easily recognized by the RES organs and to thereby remain in the circulation for longer periods of time than the “average” wild-type phage. Thus, phage interaction with the immune system is a fascinating and completely novel field of research which warrants in-depth study (Gorski and Weber-Dabrowska, 2005).

2.21 RECENT WORK IN THE FIELD OF PHAGE THERAPY

The current antibiotic resistance of most pathogenic micro-organisms together with the technical achievements in the study of phages has led to reconsider the use of bacterial viruses as a real therapeutic alternative (Levin and Bull, 1996; Allisky et al., 1998; Anderson and Levin, 1999). In Britain, Smith and Huggins (1982, 1983) carried out a series of excellent, well-controlled studies on the use of phages in systemic *E. coli* infections in mice and then in diarrhetic disease in young calves and pigs.

Bogovazova et al., (1991) studied the effectiveness of specific phage therapy on *Klebsiella* experimental sepsis in non inbred white mice, caused by intraperitoneal injection of *K. pneumoniae* K25053 into the animals. For treatment, *Klebsiella* polyvalent bacteriophage administered on day 2 after the infection of the animals with *Klebsiella* was used. The bacteriophage preparation, introduced intraperitoneally, was shown to be effective in the treatment of generalized *Klebsiella* infection. One daily intraperitoneal injection of *Klebsiella* bacteriophage 15-20 days proved to be the optimum scheme of treatment. In contrast to chemotherapeutic preparations, bacteriophage showed no effect on normal microflora and did not aggravate dysbiotic disturbances.

Soothill, (1994) examined the ability of bacteriophage to prevent the rejection of skin grafts of experimentally infected guinea pigs (Soothill, 1994). His findings demonstrated that the phage-treated grafts were protected in six of
seven cases, while untreated grafts failed uniformly, suggesting that phage might be useful for the prevention of *P. aeruginosa* infections in patients with burn wounds. Barrow and Soothill, (1997) carried out a series of studies preparatory to using phages for infections in burn patients. Using guinea pigs, they showed that skin graft rejection could be prevented by prior treatment with phages against *Pseudomonas aeruginosa*. They also saw excellent protection of mice against systemic infections with both *Pseudomonas* and *Acinetobacter* when appropriate phages were used. In the latter case, as few as 100 phages protected against infection with $10^8$ bacteria - several times higher than the $LD_{50}$.

Phage therapy has been successfully used to remove *E. coli* 0157:H7 from livestock (Barrow et al., 1998; Kudva et al., 1999; Tanji et al., 2004). A new bacteriophage CEV was isolated from the feces of sheep, naturally resistant to gut colonization by *E. coli* 0157:H7. In model system reflecting cow / sheep gut, CEV1 completely eliminated bacteria in 11 days. Phages have been also used in curing bacterial haemorrhagic ascites disease in ayu fish by *Pseudomonas plecoglossicida* (Park et al., 2000; Park and Nakai, 2002). Similarly control of eel (*Anguilla japonica*) pathogens, *Aeromonas hydrophila* and *Edwardsiella tarda* by the phages have been reported (Hsu et al., 2000).

One of the most successful studies was carried out by Biswas and coworkers (2002). Colonization of the gastrointestinal tract with vancomycin-resistant *Enterococcus faecium* (VRE), has become endemic in many hospitals and nursing homes in the United States. Such colonization predisposes the individual to VRE bacteremia and/or endocarditis, and immunocompromised patients are at particular risk for these conditions. One of these VRE strains was used to induce bacteremia in mice by intraperitoneal (i.p.) injection of $10^8$ CFU. The resulting bacteremia was fatal within 48 hours. A single i.p. injection of $3 \times 10^8$ PFU of the phage strain, administered 45 minutes after the bacterial challenge, was sufficient to rescue 100% of the animals. Even when treatment was delayed to the point where all animals were moribund, approximately 50% of them were rescued by a single injection of the phage.
Markoishvili et al., (2002) showed that bacteriophages soaked into a biodegradable film healed ulcers in 70% of 96 patients. These ulcers had not been healed by conventional treatment. Microbiological assessment was only available from 22 of the patients where healing was associated with elimination of the pathogen from the wound. The dressing also contained the antibiotic ciprofloxacin and bacteriophages commercially known as 'PyoPhage' active against strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* and *Proteus*. The healing of these wounds could not be solely attributed to the bacteriophage.

The ability of bacteriophage and their associated polysaccharide depolymerases to control enteric biofilm formation was investigated by Tait et al., (2002). Bacteriophages specific for *Enterobacter* strains were isolated from primary effluent sewage. Combination of three phages was required before complete eradication of single species biofilms of *Enterobacter cloace* occurred. Attempts to eliminate a susceptible bacterial population within a dual species biofilm were unsuccessful. It was thought that the structural heterogeneity of the biofilm produced pockets of unattainable, susceptible bacteria. These results suggest that phage and bacteria can co-exist stably within a biofilm. Bacteriophage, would, therefore, make poor tools for the control of biofilm formation. However, the results suggest that combined treatment with bacteriophage polysaccharide depolymerases and disinfectant may provide an alternative control strategy.

The protective effect of bacteriophage was assessed against experimental *S. aureus* infection in mice (Matsuzaki et al. 2003). Of the *S. aureus* phages isolated in the study, ΦMR11 was representatively used for all testing, because its host range was broad and it carried no genes for known toxins or antibiotic resistance. Intraperitoneal injections (8× 10⁸ cells) of *S. aureus*, including methicillin - resistant bacteria, caused bacteremia and eventual death in mice. In contrast, subsequent intraperitoneal administration of purified ΦMR11 (MOI >0.1) suppressed *S. aureus*-induced lethality. This lifesaving effect coincided with the
rapid appearance of ΦMR11 in the circulation, which remained at substantial levels until the bacteria were eradicated. Inoculation with high-dose ΦMR11 alone produced no adverse effects attributable to the phage.

Benedict and Flamiano, (2004) evaluated the use of bacteriophages as therapy for *Escherichia coli*-induced bacteremia in mice. Phages specific against *E. coli* were isolated from sewage and several crude preparations of the phage isolate were administered into bacteremic mice. Experiments using mouse models were first conducted to determine the minimum lethal dosage (MLD) of *E. coli* in mice. The MLD was found to be an intra-peritoneal (i.p.) injection of 0.5 ml of $10^7$ CFU/ml as it induced fatality in all replicates within 24 hours. The phage lysates, which were used in the concentrations of $10^7$, $10^8$, and $10^9$ PFU/ml, were not toxic as they induced only slight illness conditions in mice. Bacteriophage therapy experiment showed that a single i.p. injection of 0.5 ml of each of the phage lysates was enough to rescue all mice back to normal health from lethal bacteremia.

Mushtaq et al., (2005) reported that a bacteriophage encoded enzyme, endosialidase E (endo E) selectively degrades the linear homopolymeric $\alpha$-2, 8-linked N acetyleneuraminic acid capsule associated with the capacity of *E. coli* K1 strain to cause severe infection in the newborn infant. This capsular serotype is responsible for about 85% of cases of *E. coli* neonatal bacterial sepsis and meningitis. The virulent K1 strain colonized gastrointestinal tract of all 2-day old animals and produced bacteremia in over 90%. A single dose of endo E (0.25 mg) curtailed bacteremia and prevented death in at least 80% of infected animals. Older animals (up to 5 days of age) were less susceptible to systemic infection following intentional colonization. Endo-E mediated removal of K1 capsular polysaccharide led to increased ingestion by macrophages.

Vinodkumar and co-workers (2005) studied the ability of bacterial viruses to rescue septicemic mice with multidrug resistant (MDR) *Klebsiella pneumoniae* isolated from neonatal septicemia. The phage strain used in this study had lytic
activity against a wide range of clinical isolates of MDR *Klebsiella pneumoniae*. One of these MDR *Klebsiella* strain was used to induce sepsis in mice by intraperitoneal (i.p.) injection of $10^9$ CFU. The resulting bacteremia was fatal within 48 hours. A single i.p. injection of $3 \times 10^8$ PFU of the phage strain administered 45 minutes after the bacterial challenge, was sufficient to rescue 100% of the animals. Even when treatment was delayed to the point where all animals were moribund, approximately 50% of them were rescued by a single injection of this phage preparation.

The effect of phage therapy in the control of *Campylobacter jejuni* colonization in young broilers, either as a preventive or a therapeutic measure, was tested by Wagenaar *et al.*, (2005). A prevention group was infected with *C. jejuni* at day 4 of a 10-day phage treatment. A therapeutic group was phage treated for 6 days, starting 5 days after *C. jejuni* colonization of the broilers had been established. Treatment was monitored by enumerating *Campylobacter* colony forming units (CFU) and phage plaque forming units (PFU) from caecal content. Counts were compared with control birds not receiving phage therapy. A clear 3 log decline in *C. jejuni* counts was initially observed in the therapeutic group, however, after 5 days bacterial counts stabilized at a level 1 log lower than that of the control group. Colonization of *C. jejuni* in the prevention group was delayed by the treatment and after an initial 2 log reduction, colonization stabilized within a week at levels comparable to the therapeutic group. The CFU and PFU counts displayed opposing highs and lows over time, indicative of alternating shifts in amplification of bacteria and phages. There were no adverse health effects from the phage treatment. Two different phages were combined for therapeutic treatment of *Campylobacter* positive chickens and more given at the age approaching broiler harvest. This again resulted in a significant decrease in *Campylobacter* colonization concluding that phage treatment is a promising alternative for reducing *C. jejuni* colonization in broilers.

Wills and colleagues also demonstrated the efficacy with a bacteriophage against *S. aureus* in a rabbit abscess model. The sewerage-derived
bacteriophage reduced the abscess area and the count of *S. aureus* in the abscess was lowered in a bacteriophage dose dependent way (Wills *et al.*, 2005).

Marza *et al.* (2006) reported the treatment of a dog with chronic bilateral otitis external that had consistently grown *P. aeruginosa*. This infection had failed to be resolved after repeated courses of topical and systemic antibiotics. After inoculation with 400 PFU of bacteriophage into the auditory canal there was a marked improvement in the clinical signs, 27 hours after treatment.

Wang *et al.*, (2006) examined the effectiveness of phages in the treatment of imipenem resistant *Pseudomonas aeruginosa* (IMPR-Pa) infection in an experimental mouse model. 29 strains of phage were isolated from hospital sewage, and phage ΦA392 was representatively used for testing because it had lytic activity against a wide range of clinical isolates of IMPR-Pa. Intraperitoneal injections of one IMPR-Pa strain (3×10⁷ CFU) caused bacteremia and all the mice died within 24 hour. A single intraperitoneal inoculation of the phage strain (> or = 0.01 MOI) at up to 60 minutes after the bacterial challenge was sufficient to rescue 100% of the animals. This life saving effect coincided with the rapid appearance of ΦA392 in circulation (within 2 hours after injection), which remained at substantially higher levels for up to 48 hours until the bacteria were eradicated. However, the survival rates of the mice dropped to approximately 50% and 20% when the same dose of this purified phage preparation was administered at 180 minutes and 360 minutes respectively, after IMPR-Pa infection.

Use of indwelling catheters was often compromised as a result of biofilm formation. Curtin and Donlan (2006) investigated if hydrogel-coated catheters pretreated with coagulase negative bacteriophage would reduce *Staphylococcus epidermidis* biofilm formation. Biofilms were developed on hydrogel coated silicone catheters installed in a modified drip flow reactor. Catheters segments were pretreated with the lytic *S. epidermidis* bacteriophage 456 by exposing the
catheter lumen to a 10-log-PFU/ml culture of the bacteriophage for 1-hour at 37°C prior to biofilm formation. The untreated mean biofilm cell count was 7.0 ± 0.47 log CFU/cm² of catheter. Bacteriophage treatment with and without supplemental divalent cations resulted in log-CFU/cm² reduction of 4.47 (P<0.0001) and 2.34 (P=0.001) respectively. Divalent cation supplementation without bacteriophage treatment provided a 0.67 log-CFU/cm² reduction (P=0.053). Treatment of hydrogel-coated silicone catheters with the S. epidermidis bacteriophage in an in vitro model system significantly reduced viable biofilm formation by S. epidermidis over a 24 hours exposure period, suggesting the potential of bacteriophage for mitigating biofilm formation on indwelling catheters and reducing the incidence of catheter related infections.

Microflora associated with larval stages of shrimp could affect the health and development of the larvae. Some bacteria such as luminous Vibrio harveyi cause serious mortalities. Consequent to the ban on use of most antibiotics in aquaculture, there is a need for alternate technologies for control of bacterial pathogens. Bacteriophages have a potential to control bacterial pathogens. Four bacteriophages against V. harveyi were isolated, three from oyster tissue and one from shrimp hatchery water. The bacteriophages lysed 55–70% of the 100 V. harveyi isolates tested. Two bacteriophages were effective in reducing V. harveyi population in biofilm formed on high density polyethylene (HDPE) surface. In hatchery trials, bacteriophage treatment at 2×10⁶ PFU/ml level resulted in over 85% survival of Penaeus monodon larvae suggesting that bacteriophage therapy would be an effective alternative to antibiotics in shrimp hatcheries (Karunasagar et al., 2007).

Capparelli et al., (2007) reported that 10⁹ PFU of bacteriophage against S. aureus was protective against a lethal dose of S. aureus in 97% of mice when administered along with bacteria. The group also reported that mice infected intravenously with the lowest dose of S. aureus strain were not cleared by the innate immune system. But the bacterial infection could be cleared by giving the
mice a single dose of bacteriophage (10^9 PFU) 10 days after initial bacterial infection.

McVay and co-workers (2007) examined the efficacy of phage therapy in abrogating fatal *P. aeruginosa* infections in mouse burn wound model. Mice compromised by a burn wound injury and subjected to a fatal infection with *Pseudomonas aeruginosa* were administered a single dose of a *Pseudomonas aeruginosa* phage cocktail consisting of three different *P. aeruginosa* phages by three different routes: the intramuscular (i.m.), subcutaneous (s.c.), or intraperitoneal (i.p.) route. The results of this study indicated that a single dose of *P. aeruginosa* phage cocktail could significantly decrease the mortality of thermally injured, *P. aeruginosa*-infected mice (from 6% survival without treatment to 22 to 87% survival with treatment) and that the route of administration was particularly important to the efficacy of the treatment, with the i.p. route providing the most significant (87%) protection.

Watanabe *et al.* (2007) examined the efficacy of bacteriophage (phage) therapy by using a murine model of gut-derived sepsis caused by *Pseudomonas aeruginosa* that closely resembles the clinical pathophysiology of septicemia in humans. Oral administration of a newly isolated lytic phage strain (KPP10) significantly protected mice against mortality, survival rates of 66.7% for the phage-treated group versus 0% for the saline-treated control group (P< 0.01). Mice treated with phage also had lower numbers of viable *P. aeruginosa* cells in their blood, liver, and spleen. The levels of inflammatory cytokines (tumor necrosis factor alpha TNF-α, interleukin-1β [IL-1 β], and IL-6) in blood and liver were significantly lower in phage-treated mice than in phage-untreated mice. The number of viable *P. aeruginosa* cells in fecal matter in the gastrointestinal tract was significantly lower in phage-treated mice than in the saline-treated control mice.

Mice were challenged by intranasal (i.n.) inoculation with bacteria \((10^8 \text{ CFU/ml})\). A single intraperitoneal injection of \(10^9 \text{ PFU/ml}\) phage administered immediately after i.n. challenge was sufficient to rescue 100% of animals from *K. pneumoniae*-mediated respiratory infections. Administration of phage preparation 3 hours prior to i.n bacterial challenge also provided significant protection in infected mice.

The use of lytic bacteriophages to rescue septicemic mice with multidrug-resistant (MDR) *P. aeruginosa* infection was evaluated (Vinodkumar *et al.*, 2008). MDR *P. aeruginosa* was used to induce septicemia in mice by intraperitoneal (i.p.) injection of \(10^7 \text{ CFU}\). The resulting bacteremia was fatal within 48 hours. A single i.p. injection of \(3 \times 10^9 \text{ PFU}\) of the phage strain, administered 45 minutes after the bacterial challenge, was sufficient to rescue 100% of the animals. Even when treatment was delayed to the point where all animals were moribund, approximately 50% of them were rescued by a single injection of this phage preparation. The ability of this phage to rescue septicemic mice was demonstrated to be due to the functional capabilities of the phage and not to a nonspecific immune effect. The rescue of septicemic mice could be affected only by phage strains able to grow *in vitro* on the bacterial host used to infect the animals and when such strains are heat-inactivated, they lost their ability to rescue the infected mice.

### 2.22 FAILURE OF PHAGE DEFENSES

If the phages play a protective role against invading bacteria, the question arises why such phage defenses often fail? Phage occurrence may vary in a population, and phage presence and activity *in vivo* may depend upon such variable factors as the use of drugs (especially antibiotics, which may reduce the number and viability of their bacterial hosts), diet, hygiene etc. Furthermore, it has been known for many years that serum can inactivate phage via its antibody dependence and other factors (Jerne and Avegno, 1956; Cowan, 1962). Smith *et al.*, (1987) have found that anti-coliphage neutralizing antibodies may be common in human, cattle, pig and bovine serum samples, and their incidence
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may vary in relation to a specific phage. It is clear that such neutralizing antibodies may adversely influence the ability of phages to control bacteria. However, the presence of increased anti-phage antibodies in blood may also reflect their translocation rather than a phage defense response against bacteria (Gorski and Weber-Dabrowska, 2005).

Furthermore, body temperature can influence phage activity: some phages are only virulent at the highest temperatures (43°C), others being virulent at 37°C and avirulent at higher temperatures etc. Since rectal temperature in mammals can vary between 37 and 39.5°C, this phenomenon can also regulate phage functions in health and especially during infection with associated temperature elevations (Smith et al., 1987).

Another factor contributing to poor control of bacteria by endogenous phages may be the development of phage resistance by bacteria. It is likely that in vivo, the bacterium may become resistant to phages due to mutation thereby losing the receptors for phage on bacteria. Since attachment to the receptors is the initial step in the phage infection, hence such loss of receptors may influence the in vivo susceptibility of the pathogens to bacteriophages. In addition, a phage may also become resistant due to lysogeny which renders the bacterium not only immune to the original phage but also to related phages (Skurnik and Strauch, 2006). According to these workers, phage resistance may also be due to horizontal acquisition of a restriction modification system that degrade the injected phage nucleic acid or due to mutation in gene who's product is essential for phage replication assembly. These observations point toward the complexity of the phage / bacterium interactions in the animal model systems. In fact mathematical model adopted by Weld et al. (2004) to monitor phage growth in rats failed to predict their growth in vivo. However, phages can also evolve and overcome this resistance. Moreover, phage-resistant bacteria may have reduced fitness and lower ability to colonize the host. In addition, phage receptors for bacteria may correspond to their virulence determinants, such that the phages might not be able to transfer virulence factor (s) to resistant bacteria which, in
turn, will be no longer pathogenic. In other words, one could assume that in some circumstances the development of bacterial resistance to endogenous phages may be a positive phenomenon that could render the invading pathogen incapable of causing disease (Levin and Bull, 2004).

### 2.23 PHAGE THERAPY IN THE IMMUNOCOMPROMISED HOST

In humans, phages seem to be efficacious in a wide range of infections, both local and systemic, as evidenced by positive results of many clinical trials, largely from Eastern European centers (Sulakvelidze and Kutter, 2005). However, the vast majority of these studies have been performed on immunocompetent patients (Alisky et al., 1998; Sulakvelidze et al., 2001) and data on the therapeutic use of bacteriophages in the immunocompromised host are scarce. Bacterial infections, including those caused by antibiotic-resistant strains, are one of the most significant causes of morbidity and mortality in immunocompromised patients, including allograft recipients (Schmaldienst and Horl, 1997; Fishman and Rubin, 1998; Blair and Kusne, 2005; Dharnidharka et al., 2006), cancer patients (Zinner, 2000; Neuburger and Maschmeyer, 2006) individuals with primary immunodeiciencies (Carneiro-Sampaio and Coutinho, 2007) AIDS patients (Nagappan and Kazanjian, 2005; Noursadeghi et al., 2006) and burn patients (McVay et al., 2007)

One of the main questions that need to be addressed in the context of the use of bacteriophages in the treatment of bacterial infections in immunocompromised patients is regarding the general mode of antibacterial activity of phages (Parisien et al., 2008). Essentially, the in vivo therapeutic effect of a phage preparation could be mediated by either of two major mechanisms. The first relies on direct killing of bacterial cells by bacteriophage virions over the course of the lytic cycle, whereas the other depends on inducing an antibacterial immune response by either the phage particles themselves or other components of the phage preparation, especially some constituents of bacterial cells (Boratynski et al., 2004; Skurnik et al., 2007). However, several interesting experiments performed on immunocompetent mice strongly suggest that it is
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direct killing of bacteria by phage virions that is the major mechanism mediating the *in vivo* therapeutic effect of phage preparations, the induction of an antibacterial immune response playing practically no role in this regard (Biswa et al., 2002; Wang et al., 2006; Vinodkumar et al., 2008). First, a correlation was found between phage antibacterial activities *in vitro* and their therapeutic effects *in vivo*. This means that only phages capable of lysing bacterial cells *in vitro* could cure infection in mice, while those inactive *in vitro* (but potentially capable of inducing an antibacterial immune response) were ineffective *in vivo*. Interestingly, phages acting more potently *in vitro* were found to be more efficient *in vivo* (Smith and Huggins, 1982). Moreover, in a murine model of *S. aureus* bacteremia, it was shown that a mechanical lysate of *Staphylococci*, containing all the components of bacterial cells that may be present in a phage preparation, is not capable of curing infection; cure was achieved only by using a preparation containing functional bacteriophage virions (Matsuzaki et al., 2003). At the current stage of research, phage therapy appears to be a safe and effective means of treating antibiotic-resistant infections, especially in immunocompetent patients. On the other hand, data are scarce regarding the efficacy of phage therapy and the effects of bacteriophages on the immunocompromised host. Nonetheless, when combined with the results of other phage studies, these data suggest that bacteriophages may also be efficacious and safe in individuals with impaired immunity (Borysowski and Gorski, 2008).