LEVOFLOXACIN AND CEFTRIAXONE IN COMBINATION ATTENUATES LUNG INFLAMMATION IN A MOUSE MODEL OF BACTEREMIC PNEUMONIA BY MULTI-DRUG RESISTANT Streptococcus pneumoniae VIA INHIBITION OF CYTOLYTIC ACTIVITIES OF PNEUMOLYSIN AND AUTOLYSIN

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Running title: Combination therapy for drug resistant S. pneumoniae.

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Abstract

Objectives: Whether synergistic antimicrobial combination in vitro would be beneficial in down regulation of pneumococcal virulent genes and associated inflammation of the lung tissue induced by multi-drug resistant *S. pneumoniae* infection in vivo needs to be elucidated for considering this mode of therapy in case of severe pneumococcal infection.

Methods: We investigated the in vivo changes in expression of these virulence determinants using an efficacious combination predetermined by in vitro studies. Balb/C mice were infected with 10⁶ CFU of bacteria. Intravenous levofloxacin at 150 mg/kg and/or ceftriaxone at 50 mg/kg were initiated 18 h post infection; animals were sacrificed from 0 – 24 h after initiation of treatment. Levels of cytokines, chemokines and CRP in serum and in lungs, along with myeloperoxidase, nitric oxide, inflammatory cell count in broncho alveolar lavage fluid (BALF), changes in pneumolysin and autolysin gene expression and COX-2 and iNOS protein expression in lungs were estimated.

Results: Combination therapy down regulated inflammation and promoted bacterial clearance. Pneumolysin and autolysin expression was down regulated with concomitant decrease in expression of COX-2 and iNOS in lung tissue.

Conclusion: Thus this combination can be considered for therapeutic use even in cases of pneumonia caused by drug resistant isolates.

Keywords: *Streptococcus pneumoniae*, quinolones, β-lactam antibiotics, pneumolysin, autolysin, inflammation.
Streptococcus pneumoniae (SP) is the most frequent isolate from clinical samples of respiratory tract infection, including acute exacerbation of chronic bronchitis, bacteremic pneumococcal pneumonia and community acquired pneumonia (CAP) (1, 2). Despite advances in prevention by vaccination, microbiological diagnostics and antibiotic therapy, pneumonia is still characterized by a high mortality and morbidity and is associated with significant health cost (3). Treatment of CAP continues to be a challenge in 21\textsuperscript{st} century. Issues for treatment recommendations include the emergence of antibiotic resistance among SP and mono versus combination antibiotic therapy. Combination therapy should be defined as the combination of two of the following antibiotics: β-lactam, macrolide or fluoroquinolone.

A series of recent studies has cast serious doubt on whether monotherapy is appropriate in severely ill patients. Despite similar aspects of activity and favorable resistance patterns against CAP pathogens, emerging evidence suggests the superiority of dual therapy over monotherapy for certain populations, particularly patients with severe CAP or bacteremic pneumococcal CAP (4-5). Combination therapy by antimicrobials with different mechanisms of action has been used to treat infections for decade with the goal of producing a wider spectrum of action, preventing the emergence of multi drug resistant (MDR) populations, reducing the dose of a single agent or achieving a synergistic effect (6). Combination antibiotic therapy lowers mortality among severely ill patients with pneumococcal bacteremia (7, 8). Data from retrospective analyses of patients with bacteremic pneumococcal pneumonia suggest that combination antibiotic therapy is associated with reduced mortality as compared with that seen among those who receive monotherapy only (9). Combinations of antibiotics are usually adopted with the double aim of widening coverage and increasing antibacterial activity, limiting the occurrence and spread of resistant strain (10). A highly
bactericidal antibiotic combination with excellent tissue penetration, which does not lead to emergence of resistance, would be a major advantage in the treatment of pneumococcal diseases particularly for the most severe forms.

Antimicrobial therapy remains a controversial issue to treat CAP. Treatment with a single antibiotic such as a β-lactam or a third generation cephalosporin or an anti-pneumococcal fluoroquinolone was considered to be effective previously (11), but with the emergence of multi-drug resistant *S. pneumoniae* (MDRSP) strains, use of β-lactam or a macrolide as empirical therapy for such infections is of great concern. Emerging evidence shows beneficial effects on outcome with combination therapy, especially with that of a β-lactam agent and a macrolide given together to sick hospitalized patients with pneumococcal pneumonia (12). Fluoroquinolones with increased activity against SP such as levofloxacin, moxifloxacin and gemifloxacin are now being recommended. However, there has been growing concern about the emergence of fluoroquinolone resistant pneumococci (13).

If the benefit of combination therapy in patients with severe bacteremic pneumococcal pneumonia is real, something that remains to be definitively established is the mechanism in relation to lung inflammation. Until now β-lactam antibiotics have remained the drug of choice for pneumococcal diseases, except when their penetration to infected tissues is limited. Although broad spectrum cephalosporins, like cefotaxime (CTX) and ceftriaxone (CRO), which are characterized by higher levels in serum and lower MICs, have been suggested for treatment of pneumonia since 1992 (14, 15). In contrast more consistent data have been obtained in studies assessing the activities of combinations involving fluoroquinolones and β-lactams, particularly cephalosporins, both in vitro and in vivo. However, most of the cited studies limited their observations to a few strain of pneumococci and did not compare different combinations between them (16, 17). Among respiratory fluoroquinolones, levofloxacin is recommended in combination with a β-lactam (a third
Pulmonary phagocytic cell recruitment and inflammatory mediator release also play a pivotal role in the effective killing of respiratory pathogens. There has been increasing evidence indicating that during acute bacterial pneumonia the combination of bacterial virulence factors and excessive inflammatory reactions from the host together contribute to induce severe lung injury, shock, and death (20).

In this study, we investigated the antibacterial activity of combination therapy with levofloxacin and ceftriaxone in a murine model of pneumococcal pneumonia and the concomitant evolution of the inflammatory response, including phagocytic cell recruitment in bronchoalveolar lavage fluid (BALF), release of cytokines and acute phase reactive protein: C-reactive protein (CRP) in blood MPO and vascular permeability. Accumulating evidence indicates that excessive production of nitric oxide (NO) plays a pathogenic role in both acute and chronic models of inflammation (21). This has led many investigators to examine the role of inducible form of nitric oxide synthase (iNOS) and NO in pathophysiological conditions. Prostaglandins (PG), produced by cyclooxygenase (COX), are also considered as biological mediators in all stages of inflammation (22). Out of the two isoforms of COX, expression of COX-2 is rapidly induced by proinflammatory stimuli like TNF-α and is thought to be involved in inflammation. Moreover, during the inflammatory response to infection, NO and PG may work cooperatively and synergistically in both physiological and pathological conditions (23).
The high death rate associated with pneumococcal disease, the emergence of antibiotic resistance, and the problems associated with current vaccines (24) has stimulated interest in the mechanisms of virulence. Two proteins that have been implicated are pneumolysin and autolysin. Pneumolysin is a multifunctional toxin, which lyses cells with cholesterol in their membranes and interferes with the function of cells of the immune system (25). Autolysin causes cell autolysis typical of pneumococci and may be involved in cell division and competence for genetic transformation (26). Several pieces of evidence suggest that both proteins have a role in disease, (27) but the strongest has come from insertion-duplication mutagenesis experiments using the cloned genes to make pneumolysin-negative (28) and autolysin-negative (29) mutants of the pneumococcus that have reduced virulence in the mouse. Alterations in expressions of these two most important virulent factors of *S. pneumoniae* post completion of combination therapy will be crucial for understanding the effect of combination therapy on mechanism of bacterial pathogenesis in the host.

The aim of this study was to investigate the potential synergy between antibiotics of therapeutic use against *Streptococcus pneumoniae*, having broad-spectrum activity, excellent tissue penetration and less toxic to host tissue with some secondary anti-inflammatory properties against multi-drug resistant pneumococci *in vitro* and in experimental pneumonia. We have demonstrated that a combination of ceftriaxone and levofloxacin was very efficacious in experimental pneumonia particularly by down regulating pneumolysin and autolysin expression and lung inflammation. Therefore, selections of such antibiotics for combination against SP may result in synergism and should be considered for therapy.

**Materials and Methods**

**Antimicrobial agents, media and challenge organisms**

The study drugs which included penicillin (PEN), ampicillin (AMP), azithromycin (AZM), amoxicillin/potassium clavulanate (AMC), oxacillin (OXA), ceftazidime (CAZ), cefotaxime...
(CTX), cefuroxime (CXM), ceftriaxone (CRO), clindamycin (CLI), imipenem (IPM), meropenem (MEM), levofloxacin (LVX), ciprofloxacin (CIP), rifampicin (RIF), vancomycin (VAN), trimethoprim/sulphamethoxazole (TMP-SXT), cefepime (FEP) and gentamicin (GEN) (HiMedia, Bombay, India), were used for all in vitro testing and the same LVX and CRO were used for intravenous injection in mice. The clinical isolate of S. pneumoniae, AMRI-SP-1, used for the experiment was obtained from the sputum of a patient with lower respiratory tract infection, admitted to Advanced Medicare and Research Institute (AMRI) hospital in Kolkata, West Bengal, India. A quality control strain of S. pneumoniae, ATCC 49619 was obtained as a kind gift from Dr. Indranil Roy, The Calcutta Medical Research Institute (CMRI), West Bengal, India. The strains were stored in skimmed milk tryptone glucose glycerol (STGG) medium (HiMedia, Bombay, India) at -80°C and subcultured twice onto Columbia blood agar plates (BAP) supplemented with 5% sheep blood (BioMe´rieux, Lyon, France) overnight at 37°C in 10% CO₂ air incubator before use in all in vitro and in vivo experiments. All in vitro experiments were carried out in Mueller Hinton broth (MHB) (HiMedia, Bombay, India). Brain heart infusion broth (BHI) (HiMedia, Bombay, India) was used as the medium for pneumococcal cultures prior to experiments with mouse. All experimental samples were placed on Columbia BAP supplemented with 5% sheep blood.

**In vitro susceptibility tests**

In vitro susceptibilities of the isolates were compared with S. pneumoniae ATCC 49619 as per NCCLS guidelines. Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined by the tube dilution method in MHB (HiMedia, Bombay, India) supplemented with 5% sheep blood and disk agar diffusion (DAD) test was performed using Mueller Hinton agar supplemented with 5% sheep blood (30).

**Test for synergism**
Checkerboard assay was performed according to the method described earlier (31). For each combination, a synergy test was performed in a 96-well microtitre plate containing two antimicrobial agents in 2-fold dilutions (4×MIC, to 1/32 x MIC) dispensed in a checkerboard fashion on the day of the assay. \( \sum \) FICs were calculated according to the method described earlier (32). \( \sum \) FICs in each well was used to classify the effects of combinations of antimicrobial agents as: synergistic, for FIC indexes \( \leq 0.5 \); no interaction, for FIC indexes \( >0.5 \)–\( 4 \); and antagonistic, for FIC indexes \( >4 \).

**Time–kill assay**

Time kill assay was performed according to the method described earlier, (33) with little modification. Each antibiotic was tested alone and in combination at concentrations to which they showed synergy in the checkerboard assay. Bacterial counts were performed at 0, 1, 2, 4, 6, 8, 12 and 24 h of incubation at 37º C by plating aliquots of 10 and 100 µl after dilution in sterile saline (0.9%) onto Columbia BAP supplemented with 5% sheep blood. Synergy, indifference and antagonism between the combining antibiotics were concluded from methods described earlier (33).

**Lung infection model**

All experiments involving animals were conducted according to the protocols that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of Committee for the purpose of control and supervision of experiments on animal (CPCSEA) [Approval Number: 820/04/ac/CPCSEA dated: 06.08.2004], Ministry of Environment and Forest, Govt. of India. This study did not involve any invasive study using human subjects. Male Balb/C mice (25±2 g) were obtained from registered animal suppliers to the Department. All animals were maintained and utilized in accordance with recommendations from the IAEC and were provided with food and water.
ad libitum. Before infection, bacteria were grown statically at 37°C in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY broth) to OD\textsubscript{600} 0.25 (equivalent to approximately 1x10\textsuperscript{8} c.f.u. ml\textsuperscript{-1}). Experimental pneumonia was induced in the animals with a penicillin resistant (PEN, MIC >64 mg/L) strain of \textit{S. pneumoniae} AMRI-SP-1 also resistant to macrolide (AZM, MIC 8 mg/L) and fluoroquinolone (LVX, MIC 16 mg/L). Mice were anesthetized lightly by intravenous injection of ketamine hydrochloride (Sigma, Life Science) at 1 mg/kg of body weight through the tail vein, and 100 µl of a bacterial suspension (containing approximately 10\textsuperscript{7} colony forming units) was inoculated through the nares into the lungs of each mouse (50 µL per nostril). The advantage of intranasal inoculation is to mimic oropharyngeal aspiration, effectively infects upper and lower respiratory tract and is very simple. The challenge dose was confirmed by serial dilution and plating of the inocula on blood agar. Previously we had established that mice infected intranasally with 1x 10\textsuperscript{7} bacteria developed pneumonia within 18 hours from the time of infection and thus 18 h post infection time point was chosen to initiate antibiotic therapy (34).

**Treatment regimens**

18 hours after bacterial inoculation, groups of mice were treated with multiple intravenous (through tail vein) doses of either LVX (150 mg/kg body weight) or CRO (50 mg/kg body weight) only as monotherapy or administered both as combination therapy in a 0.1 mL volume with an interval of 6 hours in between two successive doses such that at the end of 24 hours each group of animal received 4 doses of the drugs either alone (monotherapy) or in combination. Animals were sacrificed for sample collection at the previously stated time point, starting at 18th hour (0h post antibiotic treatment) and continuing till 42\textsuperscript{nd} h (24\textsuperscript{th} h post antibiotic treatment) with an interval of at least 2 hours in between two successive sampling time points. Mice receiving combination therapy received 0.1 mL of LVX, immediately followed by 0.1 mL of CRO. These dosing intervals were chosen so as to simulate the \textit{in vivo}
efficacy of short term high dose treatment of the drugs in humans. Untreated *S. pneumoniae* infected animals were considered as control and were administered same volume of isotonic saline.

**Collection of lungs and blood samples**

Animals were sacrificed under ether anesthesia, starting at 18th h post infection (zero hour post antibiotic treatment) and continuing till 24 h post antibiotic therapy either single or in combination. Blood (0.5 ml) was obtained at zero hour (immediately after administration of the first dose of the drug) and also at 2, 4, 8, 10, and 24 hours (18-42 hours post infection) post antibiotic treatment by cardiac puncture under ether anesthesia and exsanguinated at those selected intervals. Before perfusion, a sample of blood so collected was placed on ice until further use in heparinized tubes and the rest part was collected in anticoagulant free tubes, kept at 37°C for half an hour before centrifuging at 3000 rpm for 15 minutes to obtain the serum. Serum thus obtained was stored in separate tubes and stored at -80°C until further use. Mice were subsequently perfused with sterile PBS to remove blood-borne bacteria from the lungs. Lungs were removed and homogenized on ice in 2 ml sterile PBS using a tissue homogenizer. To separate pneumococci from host cells, lung homogenates and blood samples were centrifuged at 855 g for 6 min at 4°C, as described previously (35). Lung and blood supernatants were subsequently centrifuged at 15,500 g for 2 min at 4°C, and the bacterial pellet was stored at -80°C until further processing. Prior to pelleting harvested bacteria, 20 µL was removed, serially diluted in PBS and plated onto blood agar in order to enumerate pneumococci present in the sample, and to determine the presence, if any, of contaminating microflora. Blood plates were incubated at 37°C in 95% air/5% CO₂ overnight. The numbers of CFU were determined by counting the numbers of single colonies that appeared on the plates showing alpha hemolysis (a characteristic specific to *S. pneumoniae*).

**Extraction of RNA from bacteria**
RNA was isolated from bacterial pellets with acid-phenol/chloroform/isoamyl alcohol (125: 24: 1, pH 4.5; Ambion, catalogue no. AM9722) essentially as described previously (35). The extract was then precipitated at -80°C overnight in the presence of 40 ng glycogen µL⁻¹ (Sigma G1767). Subsequently, the preparation was treated with 10 U RNase free DNase (Roche) at 37°C for 30 min in the presence of 1 U µL⁻¹ recombinant RNasin ribonuclease inhibitor (Promega N2511), after which RQ1 DNase stop buffer (Promega M6101) was added to inactivate the DNase. The purity of the RNA preparation was confirmed by one-step RT-PCR with or without reverse transcriptase, using 16S rRNA-specific primers, and the products were visualized after electrophoresis on a 2% TBE/agarose gel. In all cases, a PCR product was only seen in the presence of reverse transcriptase. RNA samples from a specific group from four to five mice were pooled, based on the number of c.f.u. recovered and also on the absence of contaminating bacteria, and then purified further using a Qiagen RNeasy minikit (Cat. No: 74104). RNA obtained from lung homogenates was further enriched for prokaryotic RNA using the MICROBEnrich kit (Ambion AM1901). The amount of RNA recovered following purification/enrichment was determined by OD₂₆₀/₂₈₀ measurements.

Linear amplification of mRNA

Previously, in vivo RNA studies for bacteria such as S. pneumoniae have been restricted by the amount of bacteria harvested from the animal, and therefore the yield of RNA obtained. This problem was circumvented by using an advanced, RNA linear amplification kit, MessageAmp™ Premier RNA amplification kit (Ambion, USA Catalogue No: 4383452), which employs terminal transferase to synthesize a poly-T tail onto prokaryotic cDNA. Linear amplification is then driven by a T7 phage promoter, which is incorporated at the end of a synthetic poly-A primer. A second round of amplification was performed for all lung samples in order to obtain sufficient quantities of RNA for analysis by semi-quantitative RT-PCR. Analysis of the data obtained indicated a high correlation coefficient, and no significant
difference was obtained between the amplifications, when subjected to Student’s t test (data not shown).

cDNA synthesis using Reverse Transcriptase PCR (RT-PCR)

Sufficient quantity of RNA thus obtained were used to synthesize first strand cDNA by using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific) [Lot # 00110899 #K1632]. The 20 µl of the reaction mixture according to the manufacturer’s instruction, contained 11 µl of RNA templates, 1 µl of primers (equivalent to 15 to 20 pmol), 4 µl 5x reaction buffer, 1 µl of Ribolock RNase inhibitor (20 U/µl), 2 µl of 10 mM DNTP mix and 1 µl of reverse transcriptase enzyme (200U/µl). The nucleotide sequence of the forward primer for the pneumolysin gene (ply) was 5´-TGC AGA GCG TCC TTT GGT CTAT-3´; the sequence for the reverse primer was 5´-CTC TTA CTC GTG GTT TCC-3´ (36). The nucleotide sequence of the forward primer for the autolysin gene (LytA) was 5´-ACGCAATCTAGCAGATGAAGC-3´ and the sequence for reverse primer was 5´-TGTTTGGTTGGTATTCGTGC-3´ (37). The reaction was started by an initial incubation at 42°C for 30 min and denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 15 s, 55°C for 30 s and 68°C for 1 min and a final extension at 68°C for 10 min (38). The products were stored at -80°C until further used. PCR products were run on 2% agarose gels containing 0.01% ethidium bromide (EtBr) and viewed in a BioRad GelDoc Imager. Images were analyzed using Image Lab software from BioRad laboratories.

Inflammatory cells

Leukocyte recruitment to alveoli was determined in the bronchoalveolar lavage fluid (BALF). Briefly, animals were sacrificed under ether anesthesia and trachea was exposed and intubated with a catheter before perfusion, and then repeated 1 ml injections of PBS were made until a total of 3 ml of BALF was recovered. BALF was centrifuged at 3,400 g for 10 min, and supernatant was frozen at -80°C until analysis of inflammatory mediators. Cells in
the pellet were resuspended in PBS for quantification of leukocytes with a haemacytometer, and cell populations were enumerated from Diff-Quik Stain kit (Catalog No: NC9943455; Thermo Fisher Scientific Inc.) cytospin preparation (39).

**Pharmacokinetic and pharmacodynamic studies**

The pharmacokinetic profiles of LVX and CRO were examined in parallel in both infected untreated control and infected antibiotic treated Balb/C mice.

**Bioassay.** Concentrations in lungs and serum were determined after single i.v doses of 150 mg of LVX and 50 mg of CRO per kg. In AMRI-SP 1 infected mice, antibiotic therapy was initiated at 18 h post-infection (40, 41). At 0 (5 min after drug administration), 2, 4, 8, 10 and 24 h following drug administration, three animals per group were sacrificed under ether anesthesia and blood was collected by cardiac puncture. Blood samples were centrifuged to isolate serum, pooled and frozen at -80°C until assay. Blood-free lungs were isolated and harvested from exsanguinated mice, washed in sterile sodium chloride solution, and frozen. On the day of assay, organs were weighed, pooled and homogenized in 1 mL of potassium phosphate buffer (50 mM, pH=6) at 4°C. Homogenates were centrifuged at 3000g for 30 min and the supernatants were used to quantify LVX and CRO concentrations. Drug concentrations were determined by the agar well diffusion method, using *Bacillus subtilis* (ATCC 6633) as a test strain for LVX (42) and *E. coli* (ATCC 25922) for ceftriaxone (43) as the bioassay organism. Standard curves were determined with solutions of LVX and CRO in phosphate buffer for serum and tissue in order to evaluate the active fractions of the drugs. Results were expressed as mg/L of blood or per gram of lung tissue. Standard curves were prepared in normal sera and lung homogenates from untreated mice. The standard curve was linear from 0.125 to 32 mg/L. The sensitivity of the assay was about 0.1 mg/L of sample, and the coefficients of between- and within day variations (n=3) were ≤ 7.5% at 0.5, 1, 7.5 and 20 mg/L.
Protein binding in serum

We have assumed that unbound or free drug equilibrates with the extra vascular space and that the total concentration of antibiotic in any given space is a combination of the free and protein bound drug has been considered for binding of protein in serum. Moreover the actual levels of free drug changes very little with alterations in binding to serum proteins of as much as 80% or 90%. Thus the total concentration of antibiotic in serum has been estimated for studying the in vivo efficacy of the therapy (44).

Analysis. Non-compartmental analyses were performed by routine graphical methods, for the pharmacokinetic parameters and were estimated by standard methods (45). The elimination rate constant (K_{el}) was first estimated from the slope obtained by least-squares regression analysis for the terminal portion of the concentration versus time curve. The elimination half-life (t_{1/2}) was then calculated according to the formula \( t_{1/2} = \ln 2 / K_{el} \). C_{max} (mg/L), the peak plasma concentration of a drug after administration of a dose was extrapolated from the curve. Trapezoidal rule was used to determine area under the concentration–time curve for 24 h (AUC_{0–24}); this value was divided by the MIC of the study isolate to determine the AUC_{0–24} to MIC ratio (AUC/MIC). Peak concentration to MIC ratios (peak/MIC) was also calculated for the isolate. Total serum clearance (CL) was calculated as dose/AUC. The degree of penetration into the lung tissue was determined by comparison of the area under the concentration-time curve for the lung tissue with that for the serum. Among the pharmacodynamic (PD) parameters assessed were the AUC/MIC ratio, T_{\text{S MIC}}, defined as the time period during which the serum antibiotic concentration remains above the MIC level measured in hours; and C_{max}/MIC, the ratio of maximum achievable concentration of the drug in serum to MIC.

Survival rate study
Determination of the efficacy of combination antibiotic therapy against pneumococcal pneumonia was first established in survival rate studies. Groups of mice (n=21) were inoculated intranasaly with S. pneumoniae strain AMRI SP-1 as described before. Treatments with LVX at 150 mg/kg body weight and CRO at 50 mg/kg body weight either alone or in combination by intravenous route (through the tail vein) were initiated 18 hours post infection (p.i.). Control mice received sterile saline. Survival rate was recorded every 6 hours until day 3 (72 hours) post infection.

**MPO activity as a marker of neutrophil infiltration**

Myeloperoxidase (MPO) enzyme activity was analyzed as index of neutrophil infiltration in the lung tissue, because it is closely related with the number of neutrophil present in the tissue. Blood free lung homogenates was homogenized and centrifuged at 3,000 x g for 30 minutes at 4°C. MPO activity was estimated against a standard curve made with commercially available MPO, by methods previously described (20). MPO activity from the non-infected and LVX treated mice was also estimated from a separate set of experiments.

**Estimation of nitric oxide (NO) production**

Concentration of nitrite in the lung tissues was measured as an index of NO production. Equal weights of the lungs of control (infected only) mice, infected and antibiotic treated either alone or in combination were homogenized in sterile PBS (1ml). Supernatants were collected, and analyzed for NO production by modified Greiss method as described earlier (46). Briefly, nitrate was converted to nitrites with β-nicotinamide adenine dinucleotide phosphate (NADPH; 1.25 mg/ml) and nitrate reductase followed by addition of the Griess reagent. The reaction mixture was incubated at room temperature for 20 minutes followed by the addition of trichloro acetic acid (TCA). Samples were centrifuged, clear supernatants
were collected, and optical density was recorded at 550nm. The amounts of NO produced were determined by calibrating a standard curve using sodium nitrite.

**Lung vascular permeability**

The Evans blue permeability assay was used to quantify lung capillary permeability. Evans blue avidly binds to serum albumin and can therefore be used as a tracer for transcapillary flux of macromolecules. Evans blue (0.2 ml at a concentration of 25 mg/ml) was injected into the tail vein 30 min prior to the sacrifice of 3 mice from each group. Lungs were homogenized in 2 ml of potassium phosphate buffer. Evans blue was extracted by incubating samples in 4 ml of formamide at 60°C for 24 h, followed by centrifugation at 5,000 x g for 30 min. The concentration of Evans blue was estimated by dual-wavelength (620 and 740 nm) spectrophotometry, which allowed correction of optical densities (E) for contaminating heme pigments. Thus, the following formula was used: \( E_{620} (\text{corrected}) = E_{620} - (1.426 \times E_{740} + 0.03) \); (47).

**Sample preparation for cytokine measurement from serum**

Blood samples were transferred into micro-centrifuge tubes and allowed to clot at 4°C followed by centrifugation at 3000 x g for 5 min at 4 °C. The supernatant pale yellow colored serum was pipette out carefully with the help of micropipettes into fresh micro centrifuge tubes, labeled and used for cytokine analysis. Serum from different groups were normalized to the protein content by Bradford method before the assay and levels of cytokines (IL-6, IL-10, TNF-α and IFN-γ) were determined by Sandwich ELISA according to the manufacturer’s instruction (Ray Biotech) in a Bio-Rad ELISA Reader. Serum cytokine levels were expressed in pg/ml of serum analyzed. For each study, cytokine levels were determined, in duplicate, in a single run to avoid inter-assay variability, and intra-assay variability was lower than 10-
12%. The minimum detectable dose of the cytokines for IL-6, IL-10, TNF-α and IFN-γ were 2388 pg/ml, 45 pg/ml, 60 pg/ml and 5 pg/ml respectively.

**Cytokine levels in Lung**

For cytokine (IL-6, IL-10, TNF-α and IFN-γ) measurements, lung homogenates were lysed in lysis buffer pH 7.4 consisting of 300 mM NaCl /L, 15 mM TRIS/L, 2 mM MgCl₂/L, 2 mM Triton X-100/L, 20 ng pepstatin A/mL, 20 ng leupeptin/mL, and 20 ng aprotinin/mL, and were centrifuged at 2,900 x g for 15 min at 4°C; the supernatant was frozen at -20°C, until cytokine measurement by ELISA as per manufacturer’s protocol (Ray Biotech). Normalization of lung tissue homogenate before cytokine assay was performed by determining the protein content of the homogenate using Bradford Method. Then the samples were loaded in the well of the ELISA plate having equal protein content.

**Serum C-reactive protein (CRP) estimation**

Serum CRP concentration was estimated using an ELISA kit manufactured by GenWay Biotech, Inc. San Diego, CA, USA, following the manufacturer’s instructions. Serum CRP level from the non-infected and LVX treated mice was also estimated from a separate set of experiments.

**Expression of Cox-2 and iNOS in lung tissue**

Expression of cyclooxygenase-2 (cox-2) and inducible nitric oxide synthase (iNOS) in lung tissues was determined by immunoblotting by methods described elsewhere (48). Briefly, protein levels in the tissue homogenates were estimated by the Bradford method. Twenty micrograms of each sample was electrophoresed on polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 7% skimmed milk, the blots were incubated
overnight at 4°C with primary antibodies against Cox-2 (1:1000; Chemicon, USA) and iNOS (1: 1000; Santa Cruz Biotechnology, USA). After extensive washes with PBS-Tween, blots were incubated with appropriate secondary antibodies conjugated with peroxidase (Vector, Laboratories, USA). The blots were again washed in PBS-Tween and processed for development using chemiluminiscence reagent (Millipore, USA). The images were captured and analyzed using Chemigenius, bioimaging system (Syngene, Cambridge, UK). The blots were then stripped (30 min at 50°C in 62.5 mmole/l Tris-HCl, pH 6.8, 2 % sodium dodecysulphate, and 100 mM ß-mercaptoethanol) and reprobed with anti-ß tubulin (Santa Cruz Biotechnology, USA) to determine equivalent loading of samples.

**Statistical Methods**

The observers involved in data collection and analysis were not completely blind to treatment conditions. However, the methodology used for sample identification prevented subjective bias in the experiments. On the other hand, doses and animals were randomized to treatment conditions. Data was expressed as mean ± S.D. Survival rate data were analyzed by the unpaired t-test. Means were compared between groups by using one-way analysis of variance (ANOVA). P <0.05 was considered significant for the two tests.

**Results**

**In vitro data**

Median MIC values for different antibiotics against the test isolates AMRI SP-1 and *S. pneumoniae* quality control strain ATCC-49619 were determined in sets of three according to the NCCLS broth microdilution technique. The results obtained from MIC, MBC and DAD of the pneumococcal isolate and the reference strain were listed in Table 1.

**Test for synergism**
Checkerboard method was performed in duplicate for the multi-drug resistant (MDR) isolate AMRI-SP 1 for all possible drug combinations that would be of therapeutic interest. Out of the listed antibiotics tested (listed in Table 1) to determine MICs, MBCs, and zone of inhibition, combination study, by using checkerboard assay, were performed and data obtained was represented in Table 2. The combinations that showed synergy ($\Sigma$FIC < 0.5) with the pneumococcal isolate were as follows: ciprofloxacin and cefepime > gentamicin and ceftriaxone > levofloxacin and ceftriaxone > ampicillin and azithromycin > ciprofloxacin and ceftriaxone. Antimicrobial combinations like ampicillin with levofloxacin or gentamicin, levofloxacin plus cefepime, azithromycin plus ciprofloxacin and amoxicillin/potassium clavulanate plus rifampicin showed indifference; $\Sigma$FIC value between 0.5-4. Combinations of vancomycin with rifampicin or imipenem were found to be antagonistic in action. Combinations of levofloxacin with cefotaxime or azithromycin or rifampicin were ineffective in inhibiting the growth of the tested MDR strain AMRI SP-1 ($\Sigma$ FIC>4).

**Time kill studies**

Those combinations which showed synergistic action in checkerboard assay were used to observe their bactericidal activities using that particular antimicrobial agent(s) either alone or in combination, at a concentration from which there $\Sigma$ FIC was evaluated. Although the killing activities of all antimicrobials showed remarkable improvement when they were tested in combination, synergy was observed in case of levofloxacin plus ceftriaxone and ciprofloxacin plus ceftriaxone. Representative time kill curves which showed the most effective combination as determined from the viable cell count (CFU) was demonstrated graphically for the isolate in Fig. 1. The combination of fluoroquinolones (LVX or CIP) with a third generation cephalosporin (CRO) showed synergy as the combination(s) resulted in a greater than 2 log$_{10}$ decrease in viable count at 24 h. The time kill curve also clearly showed a large decrease in viable titre at 12 h of post-antibiotic exposure for these two combinations. A
combination of CIP with FEP was able to decrease the viable titer by less than 1 log_{10} CFU and was thus considered to be indifference in action by definition. No antagonism was observed for the chosen combinations tested in time-kill assay.

**Therapeutic efficacy of the combination therapy in experimental pneumonia**

Inoculation of mice with 10^6 CFU of *S. pneumoniae* (AMRI SP-1) resulted in 100% mortality in untreated animals within 3 days post infection. (Fig. 2) LVX administered at 150 mg/kg body weight starting at 18th h post infection followed by 3 more similar doses at an interval of 6 h in between two successive dose, was associated with ~55% survival rates where as therapy with CRO alone at 50 mg/kg body weight initiated at same time followed by similar pattern of dosing resulted in ~38% survival rate. Furthermore, treatment with both the antibiotics was associated with greater than 95% survival rates. (P<0.05) Thus combination therapy with LVX and CRO proved to be effective in increasing the survival rate.

**Bacterial clearance from lungs**

Results with the AMRI SP-1 strain are represented in Fig. 3(a). 4 hours after the first intravenous dose (LVX at 150 mg/kg; CRO at 50 mg/kg), the intrapulmonary decrease in bacterial colony count was significant in the combined antibiotic treated group compared to monotherapy with LVX or CRO alone and also with the infected control group. (P< 0.05)

Moreover, subsequent multiple doses at an interval of 6 hours showed a prolonged antibacterial effect with combination therapy whereas in case of monotherapy decrease in CFU count showed no significant difference with respect to control. (P<0.05)

**Clearance of bacteremia among survivors**

Combination therapy was able to clear bacteria from the blood significantly compared to control (infected but non-treated) and monotherapy group after 4 h from the initiation of
antibiotic treatment. (P<0.05) Multiple doses of the combination eradicated almost all pathogens in circulation more effectively compared to monotherapy with either LVX or CRO and the effect was significant till the end of the experiment. Fig. 3(b).

**Analysis of bronchoalveolar lavage fluid (BALF)**

Leukocyte recruitment to alveoli was determined in the BALF. Antibiotic therapy either alone or in combination exhibited steady drop in PMN counts in BALF at every time point of the experiment compared to SP infected untreated control group. Furthermore combination therapy was more effective in decelerating PMN counts than monotherapy. A significant decrease in PMN recruitment occurred from 4 hours after initiation of therapy which corresponds to a gradual cure from bacterial invasion. As for the monocyte/macrophage recruitment in alveoli (BALF), a gradual increase was noted in untreated infected mice. A significant reduction in those cell counts was observed at 4 hours to 24 hours after initiation of treatment compared to either of the antibiotics alone. Figure 4 (a) and (b).

**Pharmacokinetics and pharmacodynamic studies from serum and lungs**

The concentration-time curves for LVX and CRO in serum and lungs were determined (data not shown). Following a single intravenous bolus administration of LVX at 150 mg/kg and CRO at 50 mg/kg body weight, the PK and PD values obtained from analysis of the serum and lungs of mice infected with *S. pneumoniae* AMRI-SP1 is shown in Table 3. Single intravenous administration of high dose LVX resulted in a peak drug concentration in the lung tissue of 40.9 ± 5.36 mg/L which was above the high MIC value for the MDR strain AMRI-SP 1 but our regimen failed to achieve a greater **C**\(_{\text{max}}\) in serum. This fact might be attributed to the high tissue penetration ability of LVX. CRO concentration achieved in lung tissue and serum was above the MIC value for the clinical isolate tested. (P<0.05)
Neutrophil infiltration: lung tissue myeloperoxidase (MPO) enzyme activity

The activity of MPO enzyme which is an indicator for neutrophil infiltration was found to be highest in the lungs of infected animals appeared at 10 h post therapy (infected controls received sterile saline). When LVX or CRO were administered alone or in combination, it caused significant (P < 0.05) time dependent reduction in tissue MPO enzyme activity compared to non-treated AMRI-SP1 infected mice. Combination therapy was able to lower the activity significantly after 8 h post-antibiotic administration compared to monotherapy.

Fig. 5 (a).

NO estimation from lung tissues

The amount of NO released into the lung tissue was measured and represented in Fig. 5 (b). NO production in lungs of control (untreated) mice was accelerated with time till the end of the experiment acting as a biological signal molecule that regulates release of other proinflammatory mediators. In our study, monotherapy with LVX or CRO failed to down regulate the production of NO till 10 h but a significant decrease was observed at 24 h post antibiotic therapy compared to the untreated control group (P<0.05). Combination therapy with these drugs resulted in a significant decrease in NO level at 8 h post initiation of antibiotic therapy and was successful in suppressing the release of this molecule till the end at 24 h post therapy compared to control or monotherapy with either drug. (P<0.05)

Vascular permeability

The pulmonary vascular permeability (as evaluated by Evans blue extravasations) showed higher values (P<0.05) in S. pneumoniae infected untreated mice (control) which was decreased gradually after treatment with LVX in combination with CRO at 8, 10 and 24 hours post antibiotic treatment. Monotherapy with LVX or CRO was unable to decrease
vascular permeability in lungs which represented persistence of inflammation at that infected site. Fig 5(c)

**Cytokine (IL-6, IL-10, IFN-γ and TNF-α) levels in serum after treatment with combined antibiotics in AMRI-SP-1 induced experimental pneumonia**

Serum IL-6 and IFN-γ levels decreased significantly whereas IL-10 level increased significantly 4 h post antibiotic therapy in *S. pneumoniae* infected mice compared to the untreated group (P < 0.05). Serum TNF-α levels remained steady until 10 h post therapy and then decreased steeply in combination treatment group compared to untreated controls or to monotherapy treated ones. IL-10 levels increased significantly after 4 h of antibiotic therapy and remained substantially high till the end of the experiment at 24 h. (P<0.05) Fig. 6; (a): IL-6; (b): IL-10; (c): IFN-γ and (d): TNF-α.

**Cytokine (IL-6, IL-10, IFN-γ and TNF-α) and chemokines IL-8 and MCP-1 levels in lungs after treatment with combined antibiotics in AMRI-SP-1 induced experimental pneumonia**

Release of pro-inflammatory, anti-inflammatory cytokines and chemokines were measured from 18 h post initiation of therapy till 24 h post antibiotic treatment to assess the level of inflammatory status in the lungs of *S. pneumoniae* infected mice groups. (Figure 7 (a): IL-6; (b): IL-10; (c): TNF-α; (d): IFN-γ; (e): IL-8 and (f): MCP-1.) Combination therapy was able to reduce the release of IL-6, TNF-α, IFN-γ, IL-8 and MCP-1 in the lungs at least 4 to 8 h after initiation of therapy compared to untreated controls or single antibiotic treated group. (P<0.05) IL-10 level also significantly increased in combined antibiotic treated group 4 h after initiation of treatment compared to untreated or single antibiotic treated groups and the level was maintained at high level till the end of the observation period. (P<0.05)
Estimation of Serum C-reactive protein (CRP) level

Serum CRP level increased in infected untreated group 18 h post infection and remained elevated at the end of 24 h of observation. Treatment with LVX or CRO alone was not able to down regulate the CRP release compared to the control untreated animals. (P<0.05) However, combination therapy was able to significantly reduce this acute phase inflammatory protein level at 8 h post antibiotic therapy compared to untreated control as well as monotherapy group. (P<0.05) Fig. 8.

Expression of pneumolysin (ply) and autolysin (lytA) mRNA

To study the expression of *S. pneumoniae* genes *in vivo* we first sought to assess whether expression of *ply* and *lytA* genes could be detected and amplified by RT-PCR and conventional PCR respectively. Total RNA extracted from lung samples of AMRI-SP 1 infected and monotherapy or combination therapy group were utilized as a template in RT-PCR reactions that allowed for the detection of *ply* and *lytA* message Fig. 9 (a) and (b). The current study showed a higher level of *ply* mRNA in the lungs of infected animals 18 h post infection. Combination therapy with LVX and CRO resulted in diminished expression of *ply* and *lytA* after 8 h post antibiotic treatment. *Ply* expression was not detected after 10 h from initiation of combination antibiotic therapy.

Effect of LVX and CRO treatment on cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) level in the *S. pneumoniae* infected mice lungs

Immunoblot analysis of lung tissue homogenate showed that COX-2 level was significantly increased at 18th h post-infection in case of the *S. pneumoniae* AMRI-SP-1 infected mice group, which was gradually decreased after 8 h of post antibiotic treatment with the drugs in
combination compared to untreated control as well as single antibiotic administered group
(P<0.05). Fig. 10.

Determination of neutrophil infiltration and estimation of serum CRP level from the
non-infected and levofloxacin treated mice

To delineate that LVX has some secondary anti-inflammatory role. We have estimated lung
tissue neutrophil infiltration by measuring the MPO activity and serum CRP concentration,
an acute phase inflammatory protein. Results showed decreased MPO activity and serum
CRP level indicating reduced PMN infiltration into the lungs concomitant with less acute
phase response. Fig. 11 A and B.

Discussion

In the era of emerging antibiotic resistance, pneumococci have developed several strategies to
survive the pressure of numerous therapeutic modalities. They are now able to resist β-
lactams, macrolides, tetracyclines, fluoroquinolones, vancomycins, trimethoprim either by
modifying the structure of bacterial cell wall synthesizing enzymes or by point mutation in
the genes or by activating efflux pumps to prevent intracellular accumulation of the drug
(49). The emergence of macrolide and cephalosporin tolerant strains, leading to treatment
failures in cases of bacteremic pneumonia, has jeopardized the efficacy of this antibiotic
combination, usually recommended as empirical treatment for community acquired and
bacteremic pneumonia by IDSA/ATS. Instead, they have also recommended a combination
of fluoroquinolone with β-lactams in the treatment of severe pneumonia (18). Furthermore,
the emergence of quinolone resistance during therapy might undermine the use of quinolones
as monotherapy for pneumococcal diseases (50). The aim of the present study was to
investigate the efficacy of administering a highly bactericidal combination regimen in an
experimental model of pneumococcal pneumonia in mice which was a prerequisite for the
treatment of pneumococcal pneumonia induced by a multi-drug resistant clinical isolate of *S. pneumoniae*.

This study revealed that several antimicrobial combinations could act synergistically in vitro against a multidrug-resistant *S. pneumoniae* isolate. In some in vitro studies, combinations of fluoroquinolones with β-lactams showed synergy against pneumococci, and a wider spectrum of activity when compared with the standard combinations (i.e. macrolide plus β-lactam) (51). More consistent data have been obtained in studies assessing the activities of combinations involving fluoroquinolones and β-lactams, particularly cephalosporins, both in vitro and in vivo (4, 15, 52). In our study, we observed that the combination of ceftriaxone with levofloxacin at sub-MIC concentration showed synergistic interaction against the multi-drug resistant strain which was consistent with previous findings.

Furthermore, to support our observation we demonstrated time-kill curves which are recognized means of detecting in vitro synergy or antagonism between antimicrobial combinations (53). Levofloxacin at a concentration of 0.5 mg/L (1/32 times MIC) in combination with ceftriaxone at 0.25 mg/L (1/8 times MIC) was more effective, producing a greater than 2 log\(_{10}\) kill in CFU within 12 h post exposure to the antibiotics in vitro. Since the multi-drug resistant isolate showed high level of penicillin and levofloxacin resistance but synergistic interaction when levofloxacin and ceftriaxone were present together and at sub-MIC concentration, we decided to treat infections due to this pathogen with an in vivo dose in which the ratio of levofloxacin would be approximately two to three times greater than ceftriaxone to mimic our findings of the in vitro time-killing assays and checkerboard experiments. Moreover, it has been reported that high dose, short-course levofloxacin regimen maximizes its concentration-dependent antibacterial activity, decreases the potential for drug resistance and has better patient compliance (54). Hence we hypothesized that if the
combination of levofloxacin and ceftriaxone was effective in vitro than when they were present alone (levofloxacin MIC 16 mg/L and ceftriaxone MIC 2 mg/L for AMRI-SP 1), this antimicrobial combination may be useful in treatment of infection due to this multi-drug resistant isolate. Moreover, how this combination regimen would be beneficial for the host in increasing survival rate and down regulating inflammation warranted an in vivo experimental pneumonia model with this pathogen.

In our study administration of levofloxacin at 150 mg/kg at an interval of 6 h for 24 h (12 mg/mice/day) closely relates to administration of 500 mg levofloxacin q 6 h (total dose 2000 mg/day) in adult individuals with normal renal function as approximately 80% of this drug is eliminated as unchanged drug in the urine through glomerular filtration and tubular secretion with minimal metabolism taking place (55). Ceftriaxone at 50 mg/kg at an interval of 6 h for 24 h (4 mg/mice/day) was equivalent to approximately 180 mg ceftriaxone every 6 h for 24 h (total dose 720 mg/day) in adult individuals. Thus, the unusual high dose of levofloxacin and low dose of ceftriaxone studied in our in vivo experiment are not alike as those recommended till date in treatment of infection due to multi-drug resistant or penicillin resistant S. pneumoniae and needs further in vivo experiments and PK/PD simulations before considering the dosages for therapeutic use.

We have studied the pharmacokinetic (PK)/pharmacodynamic (PD) parameters of drug serum and tissue concentration over time and area under the concentration curve and integrated these with the MIC of the microorganism. This could predict the likelihood of clinical success and pathogen eradication and may help in proper evaluation of the outcome of a therapy (56). It has been reported that the PK of levofloxacin are similar during multiple-dose regimen to those following single doses and the C_{max} and AUC increases linearly in a dose-dependent fashion (55). Since levofloxacin exhibits concentration dependent killing and prolonged persistent effects our aim was to maximize the concentration of the drug at the site
of infection. We achieved a high Peak level/MIC and AUC/MIC ratio for levofloxacin in the lung tissue which was required for extensive and faster degree of killing (57). On the other hand ceftriaxone exhibits time dependent killing and the only way to achieve greater killing is to maintain adequate drug levels at the site of infection for a longer period of time as these drugs have minimal to moderate persistent effects. We achieved a T>MIC for ceftriaxone approximately 8 hours with a single dose and hence multiple doses of ceftriaxone was required to maintain the drug concentration above MIC level for the strain during the 24 h period of post antibiotic therapy.

PK and PD parameters studied with a single intravenous dose of both the drugs suggested that the drugs were highly penetrable to lung tissue thereby mediating its effect which resulted in synergistic interactions in combination therapy. Since both the drugs were from different classes with different mode of action we have not compared their PK/PD parameters, but our study showed considerable amount of drug penetration at the site of infection from the blood stream, with longer half-life and the concentrations of both levofloxacin and ceftriaxone were well above the MIC level in the lung tissue. Higher doses of levofloxacin resulted in a slightly longer half-life which may be due to a larger volume of distribution of the drug.

Fluoroquinolones have been proposed to possess secondary anti-inflammatory properties, targeting the production of proinflammatory cytokines both in vitro and in vivo (58). Neutrophil infiltration into the inflammatory site is the hallmark of acute inflammation. Locally produced chemotactic factors are presumed to mediate the sequence of events leading to the infiltration at inflammation site. Our findings were also consistent with the existing literature because we have observed that monotherapy with either of the antibiotics were unable to down modulate the production and release of these immune mediators at the inflammation site (lungs) and blood (cytokines measured in serum). In contrast, combination
therapy successfully down regulated the level of IL-6, TNF-α, IFN-γ, IL-8 and MCP-1 in the lung tissue and increased the release of anti-inflammatory cytokine IL-10. Combination therapy also reduced the infiltration of inflammatory cells in the lungs compared to monotherapy.

It has been reported that influx of neutrophils and increase in vascular permeability during acute inflammation caused due to stimulation with pneumococci or pneumococcal cell wall leads to formation of edema resulting in impaired air exchange from the lungs (59). In our study we observed combination therapy was effective in reducing the pulmonary vascular leakage and edema compared to levofloxacin or ceftriaxone alone treated group.

C-reactive protein (CRP) is the prototypic acute phase protein considered to be a multifunctional component of the acute phase response and innate host defense machinery (60, 61). CRP displays calcium dependent binding specificity for phosphocholine (PCh) residues present on C-polysaccharide (PnC) of the cell wall of *Streptococcus pneumoniae* (62). Once CRP is complexed with a ligand such as PnC, it activates mouse complement, although not through the classical pathway (63). Complement activation protects mice from *S. pneumoniae* infection. The protection involves combined but independent effect of complement and CRP. Thus we can conclude that reduced bacterial load in the lungs and blood during combination therapy led to down regulation of serum CRP level.

The present study has shown the effects of combination therapy with levofloxacin and ceftriaxone on the expression of pneumolysin (*ply*) and autolysin (*lytA*) *in vivo*. Several studies have confirmed that pneumolysin and autolysins contributes to pneumococcal virulence and are key proteins for pathogenesis in humans and animals (64, 65). Pneumolysin is a multifunctional pneumococcal virulence factor that appears to augment intrapulmonary growth and dissemination during the early pathogenesis of *S. pneumoniae* infection. Inactivation of the *ply* gene of pneumococci results in an avirulent mutant (65). Murine
monoclonal antibodies to pneumolysin could prolong the survival of mice with pneumococcal pneumonia (66). The inhibition of pneumolysin in murine lung can therefore lead to improvements in the clinical outcome of pneumococcal pneumonia. Pneumolysin is not actively secreted but stored inside the bacterial cell. It is released during bacterial lysis, as from antibiotic treatment or by the action of the pneumococcal virulence factors lytA which in turn can be triggered by the immune system, antibiotic treatment (67) or other bacterial virulence factors (68). Moreover, pneumolysin has many immunomodulatory effects like inhibition of migration, respiratory burst, degranulation and other bactericidal effects in PMNs and monocytes (69). Autolysin degrades the pneumococcal cell wall, resulting in lysis and release of intracellular and cell wall molecules and is known to be crucial for pneumococcal virulence during pneumonia (70). The present study showed that combination therapy was effective in down regulating the expression of ply and lytA genes than compared to monotherapy with levofloxacin or ceftriaxone alone. Expressions of these virulent genes were exaggerated in AMRI-SP 1 infected group left untreated till the end of the observation period. But treatment of S. pneumoniae infected mice with CRO leads to slightly increased production of pneumolysin as observed in lane 3 compared to the only infected group. Antibiotic-mediated anti-inflammatory activity is achieved directly via antimicrobial activity and, in a few cases, by secondary anti-inflammatory properties of levofloxacin which target immune and inflammatory cells. With respect to the former, levofloxacin (LVX) due to its secondary anti-inflammatory activities may prevent the release of pro-inflammatory protein toxins from the Gram positive bacteria, as well as the production of other virulence factors. Secondly, levofloxacin may act directly on cells of the host defense system to inhibit the excessive production of several potent mediators of inflammation. Since the bacterial strain AMRI-SP 1 was resistant to both ceftriaxone and levofloxacin, treatment with these two antibiotics separately was not successful in eradication of bacteria from blood and lungs.
Moreover, in the presence of these antibiotics i.e. LVX alone or CRO alone treated group, the release of pneumolysin, a pro-inflammatory intracellular toxin, was increased by the disintegrating bacterial cells than that was observed in the absence of antibiotic treatment i.e. only *S. pneumoniae* infected group. Thus reduced expression of these genes was responsible for down regulation of pro-inflammatory cytokine release, respiratory burst, degranulation and other bactericidal effects in PMNs and monocytes.

In our previous study recently reported, we have observed that 18 h post infection, initiation of combination antibiotic therapy with β-lactam and macrolide like agents was able to down regulate infection and inflammation in an experimental pneumococcal pneumonia model with the isolate AMRI-SP 1 (34). In this study we have used ceftriaxone which participates in lyses of bacterial cell wall and release of those cell wall products to increase the inflammation at the site of infection. Secondly, levofloxacin used in this study have some secondary anti-inflammatory properties though the macrolides or macrolide-like agents possess a greater anti-inflammatory property compared to the fluoroquinolones. Thus, how combination therapy down regulated inflammation in the lungs and proved to be bactericidal thereby increasing the survival rate by more than 90%, still remains to be studied in detail, with more experiments on animal using this model. To delineate that LVX has some secondary anti-inflammatory role, we have estimated lung tissue neutrophil infiltration by measuring the MPO activity and serum CRP concentration, an acute phase inflammatory protein from the non-infected and LVX treated mice. Treatment of LVX showed decreased MPO activity and serum CRP level indicating reduced PMN infiltration into the lungs concomitant with less acute phase response.

To study these above mentioned mechanisms we estimated the presence of the proteins COX-2 and inducible isoform of nitric oxide synthase (iNOS) in the site of infection and inflammation (i.e. the lungs). It has been reported that expression of COX-2 mRNA was
significantly suppressed by aminoguanidine, a selective iNOS inhibitor; in rats infected with
group B Streptococcus induced inflammation of lung tissue (71). From our study, we
observed that infection with multi-drug resistant *S. pneumoniae* strain AMRI-SP 1
exaggerated the expression of iNOS and COX-2 18 h post infection in the lungs.
Combination therapy with levofloxacin and ceftriaxone down regulated the expression of
these proteins in lungs while monotherapy with these antibiotics failed to do so. Expression
of COX-2 is also partly regulated by the NO (nitric oxide) pathway because it has been
reported that infection with *S. pneumoniae* exacerbates inflammatory response leading to
production of several inflammatory mediators like TNF, interleukins, prostaglandins (PG)
and NO in the lungs of rats (72).

Decreased NO production in the lungs of mice adds evidence to the fact that the
combination therapy was efficacious for treatment of pneumococcal infection due to multi-
drug resistant strain. Thus elucidating these complex interactions may allow more rational
pharmacologic approaches to the treatment and prevention of the disease in which they play a
role.

Since it is usually recommended that combination therapy be continued for at least 3
days, though it has been reported that the potential benefit of combination therapy was
present for both 1 and 3 days (7), varying the time of infection or duration of drug
administration with PK/PD consideration still remains to be elucidated before considering
this regimen for therapeutic use.

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Transparency declarations

For the manuscript entitled “Levofloxacin and Ceftriaxone in combination attenuates lung inflammation in a mouse model of bacteremic pneumonia by multi-drug resistant Streptococcus pneumoniae via inhibition of cytolytic activities of pneumolysin and autolysin” by Arnab Majhi et al., authors declared that they have no conflict of interest for this manuscript towards submission in Antimicrobial Agents and Chemotherapy. The authors also state that we do not have a direct financial relation with the commercial identities mentioned in this manuscript that might lead to a conflict of interest for any of the authors.

References


regulatory cells control susceptibility to invasive pneumococcal pneumonia in mice. Plos Pathog. 8: 1-12.


64. Canvin JR, Marvin AP, Sivakumaran M, Paton JC, Boulnois GJ, Andrew PW, Mitchell TJ. 1995. The role of pneumolysin and autolysin in the pathology


Legends to the figures

Figure 1. Time-kill assay. Killing rates of levofloxacin (LVX) at a concentration of 0.5 µg/ml (1/32 times MIC), ciprofloxacin (CIP) at a concentration of 0.06 µg/ml (1/16 times MIC), ceftriaxone (CRO) at a concentration of 0.25 µg/ml (1/8 times MIC) and cefepime (FEP) at a concentration of 0.06 µg/ml (1/8 times MIC) either alone or in combination as LVX + CRO, CIP + CRO and CIP + FEP for the MDRSP strain AMRI SP-1. GC indicates growth control. Experiments were performed in triplicates and the results expressed as mean ± SD. P<0.05 was considered to be statistically significant.

Figure 2. Cumulative survival of group of mice (n=21) infected by intranasal challenge with multi-drug resistant S. pneumoniae isolate (AMRI SP-1) and treated with antibiotic(s) levofloxacin (LVX) and ceftriaxone (CRO) either alone or in combination and the untreated control. As of 18th h post-infection (pi) mice received 4 i.v administrations of LVX at 150 mg/kg and/or CRO at 50 mg/kg of body weight at 6 h interval in between two successive doses.

Figure 3. Bacterial burden in lungs (A) and blood (B) of mice infected with S. pneumoniae and receiving either single or a combined antibiotic treatment. Monotherapy or combination therapy was done with 4 simultaneous doses of levofloxacin (LVX) at 150 mg/kg or...
ceftriaxone (CRO) at 50 mg/kg body weight, or both, at an interval of 6 h in between two successive doses respectively. Mean bacterial counts (± SD) in lungs and blood at 18 h post infection and at several time points after initiation of antimicrobial therapy were shown. *: Significant difference with respect to infected (control) but untreated; $: significant difference compared to monotherapy with LVX or CRO. P value less than 0.05 was considered as significant.

**Figure 4. Analysis of broncho alveolar lavage fluid (BALF)**

Mean (± SD) neutrophil (a) and monocytes (b) counts in BALF of infected mice, treated with levofloxacin (LVX) at 150 mg/kg and/or ceftriaxone (CRO) at 50 mg/kg body weight. *: Significant difference with respect to infected (control) but untreated; $: significant difference compared to monotherapy with LVX or CRO. P value less than 0.05 was considered as significant.

**Figure 5. (a) MPO activity of lung tissue of mice after intranasal administration of S. pneumoniae (AMRI-SP 1) followed by treatment with levofloxacin or ceftriaxone alone or in combination.** MPO activity was analyzed as index of neutrophil infiltration in the lung tissue. The rate of change in absorbance was measured spectrophotometrically at 405 nm. MPO activity has been defined as the concentration of enzyme degrading 1 µM of peroxide/min at 37°C and was expressed as change in absorbance/min. mg tissue protein. The results were reproduced in three repeated experiments. Data are expressed as mean ± SD of mice per group (n=4). P value less than 0.05 was considered as significant. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.

**(b) NO production.** Data are expressed as mean ± SD of 4 mice per group. Values are expressed as microgram NO released per g tissue. P value less than 0.05 was considered as
statistically significant. *: Significant difference with respect to infected control; $:
significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered
as significant for all tests.

(c) Lung vascular permeability measurement. Pulmonary vascular permeability in S.
pneumoniae infected groups (mean ± SD for four mice). The results were reproduced in three
repeated experiments. Data are expressed as mean ± SD. P value less than 0.05 was
considered as significant. *: Significant difference with respect to infected control; $:
significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered
as significant for all tests.

Figure 6. Serum levels of IL-6 (a), IL-10 (b), TNF-α (c) and IFN-γ (d) in different groups
of mice at 0 to 24 h post antibiotic treatment. Levels of IL-6 (a), IL-10 (b), TNF-α (c) and
IFN-γ (d) in serum from S. pneumoniae infected mice untreated or treated with levofloxacin
(LVX) at 150 mg/kg and/or ceftriaxone (CRO) at 50 mg/kg after 18 h post infection were
determined by utilizing ELISA according to the manufacturer’s instruction and values
obtained were expressed in pg/ml as mean ± SD (n=3/group). AMRI-SP 1 infected animal
which were left untreated was considered as control while comparing with those treated with
LVX or CRO or both. *: Significant difference with respect to infected control; $: significant
difference compared to monotherapy with LVX or CRO. P<0.05 was considered as
significant for all tests.

Figure 7. Lung tissue cytokines and chemokines

Group of mice (n=21) were infected with AMRI-SP 1 and were monitored for the
development of pneumonia. 18 h post infection treatment with either levofloxacin (LVX) at
150 mg/kg or ceftriaxone (CRO) at 50 mg/kg body weight, 4 doses of antibiotics via tail vein
at an interval of 6 h in between two successive doses was initiated. Considering 18th hour to
be the hour zero of antibiotic treatment, animals were sacrificed at 0, 2, 4, 8, 10 and 24 h post antibiotic treatment. After administration of the antibiotics either alone (monotherapy) or in combination, lungs were homogenized and assayed for estimation of cytokines and chemokines. Levels of IL-6 (a); IL-10 (b); TNF-α (c); IFN-γ (d); IL-8 (e) and MCP-1 (f) were determined and mean ± SD of values obtained were expressed in pg/ml from triplicate experiments. Infected, *S. pneumoniae* infected and saline treated; LVX treated, *S. pneumoniae* infected and treated with levofloxacin; CRO treated, *S. pneumoniae* infected and treated with ceftriaxone; LVX + CRO treated, *S. pneumoniae* infected and treated with both the antibiotics. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.

**Figure 8. Serum C-reactive protein (CRP) concentration**

Serum CRP concentration was expressed as mean ± SD. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.

**Figure 9. Expression of pneumolysin (ply) and autolysin (lytA)**

The 100 base pair ladder in lane 1. Lane 2 shows effect of AMRI SP-1 infection (10^7 CFU per mice) on untreated control group at 18 h post infection. Lane 3 and 4 shows effect of monotherapy with either CRO (50 mg/kg) or LVX (150 mg/kg) at 24 h post initiation of antibiotic treatment respectively. Lane 5 to 8 shows effect of combination therapy on the expression of *ply* gene mRNA (a) and *lytA* gene (b) at 2, 4, 8, 10 and 24 h post therapy.

**Figure 10. Expression of COX-2 and iNOS in the lungs**
Representative results of western blot analysis of the inhibitory effects of levofloxacin (LVX) at 150 mg/kg and ceftriaxone (CRO) at 50 mg/kg four times in 24 h at an interval of 6 h in between two successive doses on the production of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by multi-drug resistant clinical isolate of *S. pneumoniae* AMRI-SP 1 in murine lungs. Lane 1 shows effect of AMRI SP-1 infection (10⁷ CFU per mice) on untreated control group at 18 h post infection. Lane 2 and 3 shows effect of monotherapy with either LVX (150 mg/kg) or CRO (50 mg/kg) at 24 h post initiation of antibiotic treatment respectively. Lane 4 to 7 shows effect of combination therapy on the expression of COX-2 and iNOS at hour zero, 4, 8 and 24 h post antibiotic therapy. (b) and (c) Signal intensity of each band and control expressed in arbitrary unit. Data present mean ± SD of four mice. *: Significant difference with respect to control (untreated); $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.

**Figure 1.** (A) MPO activity of lung tissue of mice after intranasal administration of *S. pneumoniae* (AMRI-SP 1) followed by treatment with levofloxacin or ceftriaxone alone or in combination. MPO activity was analyzed as index of neutrophil infiltration in the lung tissue. The rate of change in absorbance was measured spectrophotometrically at 405 nm. MPO activity has been defined as the concentration of enzyme degrading 1 µM of peroxide/min at 37°C and was expressed as change in absorbance/min. mg tissue protein. Data are expressed as mean ± SD of mice per group (n=4). P value less than 0.05 was considered to be significant. *: significant difference with respect to untreated infected group, $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests. (B) Serum C-reactive protein (CRP) concentration was expressed as mean ± SD. *: significant difference with respect to untreated infected group.
significant difference compared to monotherapy with LVX or CRO. $P<0.05$ was considered as significant for all tests.
TABLE 1. In vitro susceptibilities of *Streptococcus pneumoniae* strains to different antimicrobial agents* for all test isolates.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/L)</th>
<th>MBC (mg/L)</th>
<th>Zone Diameter (mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ATCC 49619</td>
<td>AMRI SP-1</td>
<td>ATCC 49619</td>
</tr>
<tr>
<td>PEN</td>
<td>0.06a</td>
<td>&gt;64b</td>
<td>0.06</td>
</tr>
<tr>
<td>AMP</td>
<td>0.25a</td>
<td>&gt;32b</td>
<td>0.5</td>
</tr>
<tr>
<td>AMC</td>
<td>0.06a</td>
<td>2b</td>
<td>0.12</td>
</tr>
<tr>
<td>OXA</td>
<td>0.06a</td>
<td>0.25a</td>
<td>0.25</td>
</tr>
<tr>
<td>CAZ</td>
<td>8c</td>
<td>16d</td>
<td>16</td>
</tr>
<tr>
<td>CRO</td>
<td>1a</td>
<td>2b</td>
<td>4</td>
</tr>
<tr>
<td>CTX</td>
<td>4a</td>
<td>0.5a</td>
<td>8</td>
</tr>
<tr>
<td>CXM</td>
<td>0.5a</td>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>FEP</td>
<td>0.06a</td>
<td>0.5a</td>
<td>0.06</td>
</tr>
<tr>
<td>IPM</td>
<td>0.06a</td>
<td>0.12a</td>
<td>0.12</td>
</tr>
<tr>
<td>MEM</td>
<td>0.06a</td>
<td>0.06a</td>
<td>0.12</td>
</tr>
<tr>
<td>AZM</td>
<td>0.12a</td>
<td>&gt;8b</td>
<td>2</td>
</tr>
<tr>
<td>CLI</td>
<td>0.5a</td>
<td>4b</td>
<td>0.5</td>
</tr>
<tr>
<td>LVX</td>
<td>0.12a</td>
<td>16b</td>
<td>0.12</td>
</tr>
<tr>
<td>CIP</td>
<td>0.06a</td>
<td>1a</td>
<td>0.12</td>
</tr>
<tr>
<td>RIF</td>
<td>0.06a</td>
<td>0.12a</td>
<td>0.12</td>
</tr>
<tr>
<td>VAN</td>
<td>1a</td>
<td>&gt;64b</td>
<td>4</td>
</tr>
<tr>
<td>GEN</td>
<td>0.5a</td>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>TMP-SXT</td>
<td>1a</td>
<td>&gt;64b</td>
<td>2</td>
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TABLE 2. Evaluation of synergy by chequerboard assay

<table>
<thead>
<tr>
<th>Combination (A + B)</th>
<th>FIC A</th>
<th>FIC B</th>
<th>∑ FIC</th>
<th>Remarks</th>
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</thead>
<tbody>
<tr>
<td>Antibiotic A Conc. A (mg/L)</td>
<td>Antibiotic B Conc. B (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td>Ampicillin 1</td>
<td>Azithromycin 2</td>
<td>0.093</td>
<td>0.037</td>
<td>0.468</td>
</tr>
<tr>
<td>Ampicillin 1</td>
<td>Levofoxacin 8</td>
<td>0.281</td>
<td>0.562</td>
<td>0.843</td>
</tr>
<tr>
<td>Ampicillin 1</td>
<td>Gentamicin 0.25</td>
<td>0.039</td>
<td>0.620</td>
<td>0.659</td>
</tr>
<tr>
<td>Levofoxacin 0.5</td>
<td>Ceftriaxone 0.25</td>
<td>0.046</td>
<td>0.375</td>
<td>0.421</td>
</tr>
<tr>
<td>Levofoxacin -</td>
<td>Cefotaxime -</td>
<td>$</td>
<td>$</td>
<td>$</td>
</tr>
<tr>
<td>Levofoxacin 0.03</td>
<td>Cefepime 0.06</td>
<td>0.035</td>
<td>1.12</td>
<td>1.159</td>
</tr>
<tr>
<td>Levofoxacin -</td>
<td>Azithromycin -</td>
<td>$</td>
<td>$</td>
<td>$</td>
</tr>
<tr>
<td>Vancomycin 2</td>
<td>Rifampicin 0.06</td>
<td>0.064</td>
<td>17.16</td>
<td>17.22</td>
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<tr>
<td>Vancomycin 16</td>
<td>Imipenem 0.12</td>
<td>0.251</td>
<td>134.33</td>
<td>134.58</td>
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<tr>
<td>Azithromycin 0.25</td>
<td>Ciprofloxacin 4</td>
<td>0.132</td>
<td>1.062</td>
<td>1.194</td>
</tr>
<tr>
<td>Ciprofloxacin 0.06</td>
<td>Ceftriaxone 0.25</td>
<td>0.312</td>
<td>0.156</td>
<td>0.468</td>
</tr>
<tr>
<td>Ciprofloxacin 0.06</td>
<td>Cefepime 0.06</td>
<td>0.124</td>
<td>0.248</td>
<td>0.372</td>
</tr>
<tr>
<td>Amoxicillin/ Potassium clavulanate 0.06</td>
<td>Rifampicin 0.03</td>
<td>0.032</td>
<td>0.531</td>
<td>0.562</td>
</tr>
<tr>
<td>Gentamicin 0.06</td>
<td>Ceftriaxone 0.12</td>
<td>0.187</td>
<td>0.187</td>
<td>0.374</td>
</tr>
</tbody>
</table>

*S*- Resistant to the combination. FIC- Fractional inhibitory concentration. ∑ FIC (FIC A + FIC B) value < 0.5, synergy; 0.5 – 4, indifference; > 4, antagonism. *; no effective inhibition in growth after 24 h of incubation at 37°C compared to O.D observed at zero hour at 550 nm.
### TABLE 3.

Pharmacokinetic and pharmacodynamic parameters\(^a\) for levofloxacin (LVX) and ceftriaxone (CRO) following a single intravenous dose at 18h post infection.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SD</th>
<th>Levofloxacin (LVX)</th>
<th>Ceftriaxone (CRO)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Lungs</td>
<td>Serum</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>150</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>C(_{\text{max}}) (mg/L)</td>
<td>9.8 ± 2.03</td>
<td>40.9 ± 5.36</td>
<td>44.5 ± 3.5</td>
</tr>
<tr>
<td>AUC(_{0-24}) (mg.h/L)</td>
<td>92.64 ± 9.08</td>
<td>146.26 ± 23.37</td>
<td>97.6 ± 11.23</td>
</tr>
<tr>
<td>t(_{1/2}) (h)</td>
<td>12.37 ± 3.21</td>
<td>12.06 ± 2.67</td>
<td>1.51 ± 0.67</td>
</tr>
<tr>
<td>CL</td>
<td>1.62 ± 0.16</td>
<td>1.02 ± 0.07</td>
<td>0.51 ± 0.05</td>
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<tr>
<td>Pharmacodynamics</td>
<td></td>
<td></td>
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<tr>
<td>AUC/MIC (h)</td>
<td>5.79 ± 1.42</td>
<td>9.14 ± 1.33</td>
<td>48.8 ± 5.61</td>
</tr>
<tr>
<td>T&gt;MIC (h)</td>
<td>b</td>
<td>16.47 ± 2.48</td>
<td>9.19 ± 2.21</td>
</tr>
<tr>
<td>C(_{\text{max}})/MIC</td>
<td>0.619 ± 0.12</td>
<td>2.56 ± 0.29</td>
<td>22.25 ± 0.25</td>
</tr>
</tbody>
</table>

\(^a\) Abbreviations: C\(_{\text{max}}\), maximum concentration of drug in serum; C\(_{\text{min}}\), minimum concentration of the drug in serum, t\(_{1/2}\), time taken to reach half of the maximum concentration, AUC, area under the concentration-time curve from time zero to 24 h post antibiotic treatment. T>MIC – Time during which the drug concentration remains above the MIC value in serum; AUC/MIC - Ratio of area under the curve during 24 h of post antibiotic treatment to the MIC value for the \(S. \text{pneumoniae}\) strain AMRI-SP1; C\(_{\text{max}}\)/MIC - ratio of maximum concentration of the drug achieved in serum to MIC of the drug.

\(-b\) Peak drug concentration achieved in serum was below the MIC of Levofloxacin (16 mg/L) for the MDR strain AMRI-SP1, but was quite high above sub-MIC value of 0.5 mg/L (1/32 times MIC) used for the time-kill assay \textit{in vitro} using the same isolate.
Figure 1. Time-kill assay. Killing rates of levofloxacin (LVX) at a concentration of 0.5 µg/ml (1/32 times MIC), ciprofloxacin (CIP) at a concentration of 0.06 µg/ml (1/16 times MIC), ceftriaxone (CRO) at a concentration of 0.25 µg/ml (1/8 times MIC) and cefepime (FEP) at a concentration of 0.06 µg/ml (1/8 times MIC) either alone or in combination as LVX + CRO, CIP + CRO and CIP + FEP for the MDRSP strain AMRI SP-1. GC indicates growth control. Experiments were performed in triplicates and the results expressed as mean ± SD. P<0.05 was considered to be statistically significant.
Figure 2. Cumulative survival of group of mice (n=21) infected by intranasal challenge with multi-drug resistant *S. pneumoniae* isolate (AMRI SP-1) and treated with antibiotic(s) levofloxacin (LVX) and ceftriaxone (CRO) either alone or in combination and the untreated control. As of 18<sup>th</sup> h post-infection (pi) mice received 4 i.v administrations of LVX at 150 mg/kg and/or CRO at 50 mg/kg of body weight at 6 h interval in between two successive doses.
Figure 3 Bacterial burden in lungs (A) and blood (B) of mice infected with *S. pneumoniae* and receiving either single or a combined antibiotic treatment. Monotherapy or combination therapy was done with 4 simultaneous doses of levofloxacin (LVX) at 150 mg/kg or ceftriaxone (CRO) at 50 mg/kg body weight, or both, at an interval of 6 h in between two successive doses respectively. Mean bacterial counts (± SD) in lungs and blood at 18 h post infection and at several time points after initiation of antimicrobial therapy were shown. *: Significant difference with respect to infected (control) but untreated; $: significant difference compared to monotherapy with LVX or CRO. P value less than 0.05 was considered as significant.
**Figure 4 Analysis of broncho alveolar lavage fluid (BALF)**

Mean ± SD neutrophil (A) and monocytes (B) counts in BALF of infected mice, treated with levofoxacin (LVX) at 150 mg/kg and/or ceftriaxone (CRO) at 50 mg/kg body weight. *: Significant difference with respect to infected (control) but untreated; $: significant difference compared to monotherapy with LVX or CRO. P value less than 0.05 was considered as significant.
Figure 5. (a) MPO activity of lung tissue of mice after intranasal administration of *S. pneumoniae* (AMRI-SP 1) followed by treatment with levofloxacin or ceftriaxone alone or in combination. MPO activity was analyzed as index of neutrophil infiltration in the lung tissue. The rate of change in absorbance was measured spectrophotometrically at 405 nm. MPO activity has been defined as the concentration of enzyme degrading 1 μM of peroxide/min at 37 C and was expressed as change in absorbance/min. mg tissue protein. The results were reproduced in three repeated experiments. Data are expressed as mean ± SD of mice per group (n=4). P value less than 0.05 was considered as significant. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests. (b) NO production. Data are expressed as mean ± SD of 4 mice per group. Values are expressed as microgram NO released per g tissue. P value less than 0.05 was considered as statistically significant. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests. (c) Lung vascular permeability measurement. Pulmonary vascular permeability in *S. pneumoniae* infected groups (mean ± SD for four mice). The results were reproduced in three repeated experiments. Data are expressed as mean ± SD. P value less than 0.05 was considered as significant. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.
Figure 6. Serum levels of IL-6 (a), IL-10 (b), TNF-α (c) and IFN-γ (d) in different groups of mice at 0 to 24 h post antibiotic treatment. Levels of IL-6 (a), IL-10 (b), TNF-α (c) and IFN-γ (d) in serum from *S. pneumoniae* infected mice untreated or treated with levofloxacin (LVX) at 150 mg/kg and/or ceftriaxone (CRO) at 50 mg/kg after 18 h post infection were determined by utilizing ELISA according to the manufacturer’s instruction and values obtained were expressed in pg/ml as mean ± SD (n=3/group). AMRI-SP 1 infected animal which were left untreated was considered as control while comparing with those treated with LVX or CRO or both. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.
Figure 7. Lung tissue cytokines and chemokines

Control of mice (n = 6) infected with M. bovis BCG and were monitored for the development of pneumonia at various time points with and without MDR-1 and IL-6 levels following treatment with control or 10 mg/kg/day of LIX, CRO or P O. S, * and ** indicate significant difference compared to monotherapy with LIX or CRO, P = 0.05 was considered significant for all tests.

- **Lung IL-6 concentration**
  - Control
  - LIX-treated
  - CRO-treated
  - P O-treated

- **Lung IL-10 concentration**
  - Control
  - LIX-treated
  - CRO-treated
  - P O-treated

- **Lung MCP-1 concentration**
  - Control
  - LIX-treated
  - CRO-treated
  - P O-treated

- **Lung TNF-α concentration**
  - Control
  - LIX-treated
  - CRO-treated
  - P O-treated
**Figure 8. Serum C-reactive protein (CRP) concentration**

Serum CRP concentration was expressed as mean ± SD. *: Significant difference with respect to infected control; $: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.
Figure 9. Expression of pneumolysin (ply) and autolysin (lytA)
The 100 base pair ladder in lane 1. Lane 2 shows effect of AMRI SP-1 infection (10^7 CFU per mice) on untreated control group at 18 h post infection. Lane 2 and 3 shows effect of monotherapy with either CRO (50 mg/kg) or LVX (150 mg/kg) at 24 h post initiation of antibiotic treatment respectively. Lane 4 to 8 shows effect of combination therapy on the expression of ply gene mRNA (a) and lytA gene (b) at 2, 4, 8, 10 and 24 h post therapy.
Figure 10. Expression of COX-2 and iNOS in the lungs
(a) Representative results of western blot analysis of the inhibitory effects of levofloxacin (LVX) at 150 mg/kg and ceftriaxone (CRO) at 50 mg/kg four times in 24 h at an interval of 6 h in between two successive doses on the production of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by multi-drug resistant clinical isolate of *S. pneumoniae* AMRI-SP 1 in murine lungs. Lane 1 shows effect of AMRI SP-1 infection (10⁶ CFU per mice) on untreated control group at 18 h post infection. Lane 2 and 3 shows effect of monotherapy with either LVX (150 mg/kg) or CRO (50 mg/kg) at 24 h post initiation of antibiotic treatment respectively. Lane 4 to 7 shows effect of combination therapy on the expression of COX-2 and iNOS at hour zero, 4, 8 and 24 h post antibiotic therapy. (b) and (c) Signal intensity of each band and control expressed in arbitrary unit. Data present mean ± SD of four mice. *: Significant difference with respect to control (untreated); $*: significant difference compared to monotherapy with LVX or CRO. P<0.05 was considered as significant for all tests.