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Original Article

Refined Intratracheal Intubation Technique in the Mouse, Complete Protocol Description for Lower Airways Models

John Sparrowe[1,*], Magdalena Jimenez[1], Joaquin Rullas[2], Antonio E. Martínez[1] and Santiago Ferrer[2]

[1] 1Laboratory Animal Science, GSK, Tres Cantos, Spain
[2] Medicines Development, DDW GSK, Tres Cantos, Spain

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Corresponding Author:
John Sparrowe
john.g.sparrowe@gsk.com

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ABSTRACT

Intratracheal intubation (i.t.) in mice is a technique required for many in vivo study protocols, as are the intranasal (i.n.) route or the use of aerosol generators. The i.n. technique is rapid to perform but erratic whereas the transtracheal route requires a short surgery, with anesthesia and a few days needed for total recovery and wound healing before the study can be performed. The i.t. route is a reliable, fast and simple technique and we provide a detailed description for intubation by transthoracic illumination and confirmation in the mouse, using commercially available tools. The result is a technique that takes about 40 seconds to perform, including verification of right positioning of the probe, with no mortality, pulmonary edema, bleeding or laryngeal damage observed. However checking the correct placement of the probe is crucial for good results. This method's robustness was evaluated by comparing bronchoalveolar lavage (BAL) cell count results, obtained both from i.n. instillation and i.t. inoculation of LPS (*E. coli* Lipopolysaccharide) to produce a model of lung lower airways inflammation to evaluate anti-inflammatory compounds. We also describe its use as the standard infection technique in an acute *Mycobacterium tuberculosis* infection model for therapeutic efficacy studies.

Keywords: Commercially available tools, intratracheal intubation, mouse, verification.

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INTRODUCTION

Both administration of substances and inoculation of pathogens in the respiratory tract are needed when carrying out research on respiratory diseases. For this purpose, in order to learn every detail about the natural route that a defined airborne pathogen follows within the host and how this pathogen will spread, mimicking nature is imperative. To achieve this, the i.n. route or an aerosol generator will be preferred (Driscoll *et al*., 2000).

The i.n. administration probably is the easiest to perform and at the same time the most imprecise of all techniques, as there is no possibility to know how much volume will stay on nasal mucosa, how much will be swallowed or how much will reach lower airways. To perform this technique the anesthetized mouse is restrained and elevated or placed on lateral

recumbence and then, by applying a pipette into its nares the inocula or compound will be administered.

For aerosol generation there are available several commercial devices. Their airflow and pressure must be adjusted and tested for each micro organism and vehicle used, prior to create adequate size droplets so they can reach lower airways following inhalation. However, not all microorganisms are viable after this process.

Once the mechanisms of infection and colonization have been elucidated, the less physiological routes and techniques that bypass the nasopharynx's natural filtering function can be developed, to achieve robust, quantitative and highly reproducible animal models. These techniques deliver the amount of substance or pathogen in a precise way, thus making available consistent responses in groups of animals. There are several techniques available, such as i.t. intubation (Costa *et al.,* 1986; Ho *et al.,* 1973), which nowadays many research groups use as a routine technique for their in vivo work protocols.

The transtracheal technique needs a small incision on the ventral aspect of the neck and a blunt dissection to expose tracheal rings. Then, there are two options, to insert a needle or to introduce a catheter through a small cut made between rings. After delivering the inocula, the incision is closed; the animals have to recover from general anesthesia and receive post operative analgesics and care. However, in studies involving large numbers of animals, this may be time consuming. A reliable, rapid and simple technique for orotracheal intubation and confirmation is desirable.

The aim of this article is to explain, step by step, an accurate and easy to learn way to successfully intubate mice, using commercially available equipment. This is a description of a technique we have been using for more than ten years with no mortalities. A method for checking the right placement of the probe, simple and safe for the animal is also included. This method was assessed and validated comparing BAL number of cell count results obtained both from i.n. instillation and i.t. inoculation of LPS. Then it was applied in a lower airway infection model where it proved to be highly successful.

We also aim to convince all those who still consider transtracheal instillation as an option to change to this less invasive alternative, thus helping progress in refinement.

MATERIALS AND METHODS

Animals

As part of various ongoing scientific programmes this technique was performed with CD1 and C57BL/6J of miscellaneous weights (Charles River Laboratories and Harlan Laboratories Models). The procedure was performed after the five days acclimatization period, using animals between 8 and 18 weeks of age. They were housed in groups of five, in type-III cages with autoclaved corncob bedding. Temperature was 21 ± 1ºC and humidity 55 ± 10%. Water (ultra filtrated) and food (Tecklad, Global Rodent Maintenance 2014, irradiated) were provided *ad libitum*. The light/dark cycle was 12:12 with lights on at 8:00 and dawn/dusk facility.

All animal studies were ethically reviewed and carried out in accordance with European Directive 86/609/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals.

Premedication and anesthesia

Animals were premedicated and anesthetized for the procedure. Premedication of the animals, by intraperitoneal injection of the following cocktail given 15 minutes before anesthesia as a standard practice, prevents any possible complication. The cocktail contains doxapram hydrochloride at 10 mg/Kg (Respiram, Modern Veterinary Therapeutics, LLC, Miami, Florida 33157 USA.) and atropine (Atropina®, B. Braun Medical SA Barcelona Spain) at 0.05 mg/Kg. Doxapram is an analeptic agent that causes an increase in tidal volume and respiratory frequency, thus helping during and immediately after the technique is done, speeding the recovery; atropine helps in decreasing respiratory and salivary secretions and

preventing bradycardia. Inhalational anesthesia with isoflurane at 3% (Forane® from Abbot Laboratories) was used to produce induction, in an anesthetic chamber. This provides a sufficient duration of anesthesia to perform the whole process. If anesthesia maintenance is needed, for example when training in the technique, an open anesthetic circuit with scavenging system, and a facemask attached to the intubation support providing isoflurane at approximately 1.3% can be used.

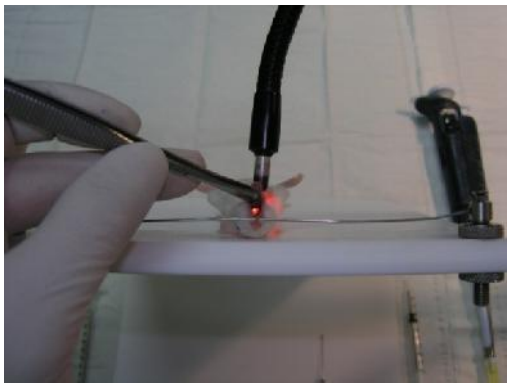


Figure 1: Intubation support

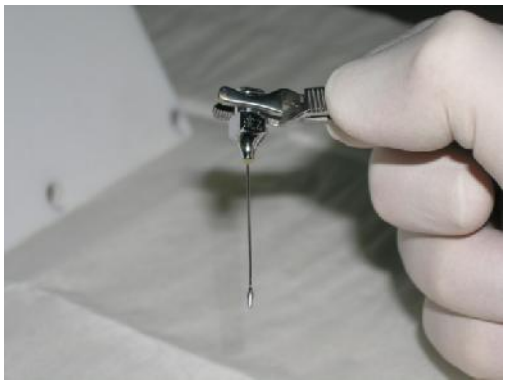


Figure 2: Bulldog serrefines handle

Materials Used

To perform this technique we used an angled support (Fig. 1), fiber-optic light, premedication cocktail, isoflurane calibrated vaporizers, specific probes with handles, Gilson P100 Automatic pipette and soap foam.

The intratracheal probe chosen was item 27.134 (Lachrymal olive Luer lock, 0.60 × 30 mm. 23G×1 1/4", Veterinary needles catalogue, from Unimed, Switzerland (Figs. 2, 3). A suitable handle was easily provided by means of Bulldog type serrefines, (curved 35 mm No.18051-35, from F.S.T. Heidelberg, Germany), applied to the Luer cone (Fig. 2). Another possibility is to ask for specific handles to be made by an instrument engineer out of stainless steel. (The one shown in Fig.3 is available at Cancela, Fax no. +34 91 845 9853 Ref. No. HM-012. Madrid Spain)

The substance and inocula administered were *E.coli* LPS in sterile saline, and *M. tuberculosis* in PBS and the final volume to be delivered in deep lung was adjusted to 40-50 µL correspondingly.

A first validation was carried out administering four groups of animals (n=5) 40 µl of lps solution by both intranasal and intratracheal routes. Bronchoalveolar lavage (BAL) fluid was obtained by washing with 1 mL sterile saline solution, in and out of the lungs three times via a tracheal cannula. Samples were centrifuged at 1000 rpm for 5 min. The cells were resuspended in PBS and counted in a BD FACSCalibur™ flow cytometer.

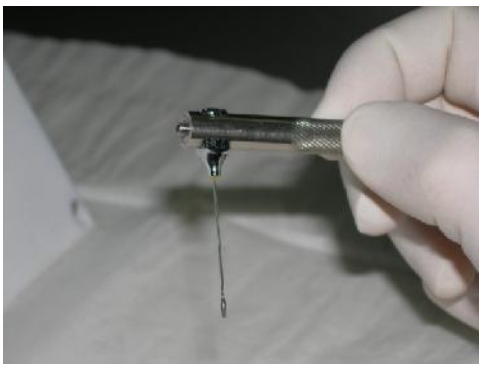


Figure 3: Commercial handle

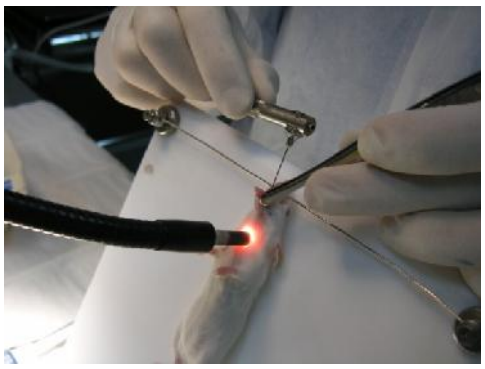


Figure 4: Accessing the airway

Following this rationale, the technique was put to the test, infecting C57BL/6 mice by non-surgical intratracheal instillation of 10^5 viable Colony Forming Units (CFU) of *M. tuberculosis*. Mice were sacrificed at day 9 after infection, lungs were aseptically obtained and, after homogenization, serial dilutions of homogenates were plated to measure the CFU burden per whole organ.

Technique Description

The first step to take, and critical for success, is to choose a proper probe. The ideal probe for direct tracheal intubation should be rigid, narrow, atraumatic and at the same time able to allow delivery of the compound or inocula. To save time, it is much preferable if there is no need to use a Seldinger wire. A rapid procedure is required to make it possible to prepare an adequate number of animals for Toxicology, DMPK or Therapeutic Efficacy studies. These probes for standard procedures should be recyclable, washable and autoclave resistant, the same way standard gavage dosing probes are. Due to the different weights, sizes and ages of mouse strains used, care should be taken in choosing a suitable item for any specific study.

Considering all the above mentioned, a biconical tip with round cross section, blunt enough to be safely maneuvered in such a narrow passage would give the solution. This biconical tip in addition must have a maximum diameter (1.26 mm) smaller than the average tracheal airway diameter (1.36 mm) (Oldham *et al.*, 2002). So, on one hand the access is granted and on the other hand it also gives the animal the chance of keeping spontaneous breathing through the space between trachea and probe.

Because of the species characteristic excessive mucus production when anesthetized, it is imperative to use this technique with ultra-short-acting anesthesia, preferably combined with the premedication cocktail described above given before the procedure. When dealing with large mice, low density of the compound or inocula to be administered, or if a slow inflammatory response is expected this premedication could be omitted. However we recommend that it should be used always when learning this technique.

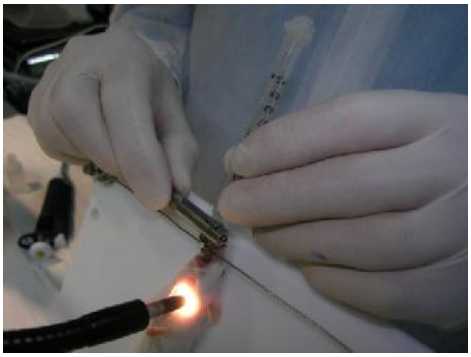


Figure 5: Intubation confirmation

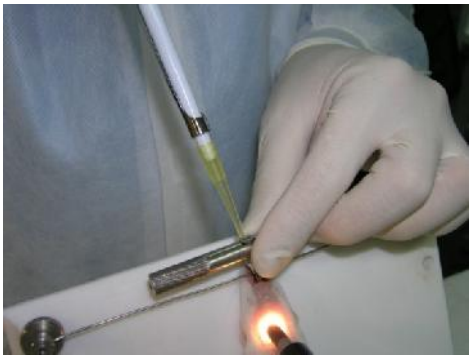


Figure 6: Delivery of inocula/compound

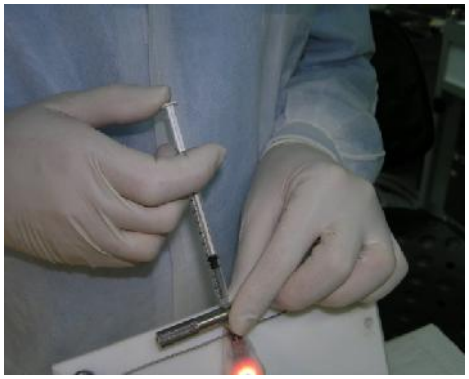


Figure 7: Air bolus administration

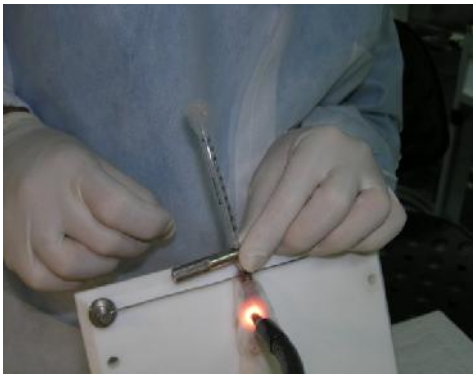


Figure 8: Final checking

Before and after administration or inoculation, while the probe is in place, a 1mL syringe barrel with a small amount of soap foam is used to check the correct positioning of the probe inside the trachea (Figs. 5, 8). The animal will easily push the foam, moving it up and down without effort. This is much more reliable than air bolus administration to move thorax or fog visualization on a glass or a mirror surface and safer than feeling tracheal rings by moving the probe against them (Esposito et al., ,1983). Checking the right placement of the probe is crucial for good results.

After checking the correct positioning of the probe the compound or inocula is delivered by means of an automatic pipette, applying its tip inside and right in the middle of the Luer cone (Fig. 6). To help progress the full volume to the trachea, three air boluses of 200 µL each (as this is the average tidal volume in mice (Brown *et al*., 1999), will be administered by means of a one mL syringe (Fig. 7). This way we facilitate recovery, assure to deliver completely the administration/inoculation and also clean the probe for the final checking of good positioning (Fig. 8).

Validation Results

Before implementation of this method as a routine technique, its robustness was assessed and validated by comparing BAL number of cells count results obtained from intranasal instillation and intratracheal inoculation of LPS.

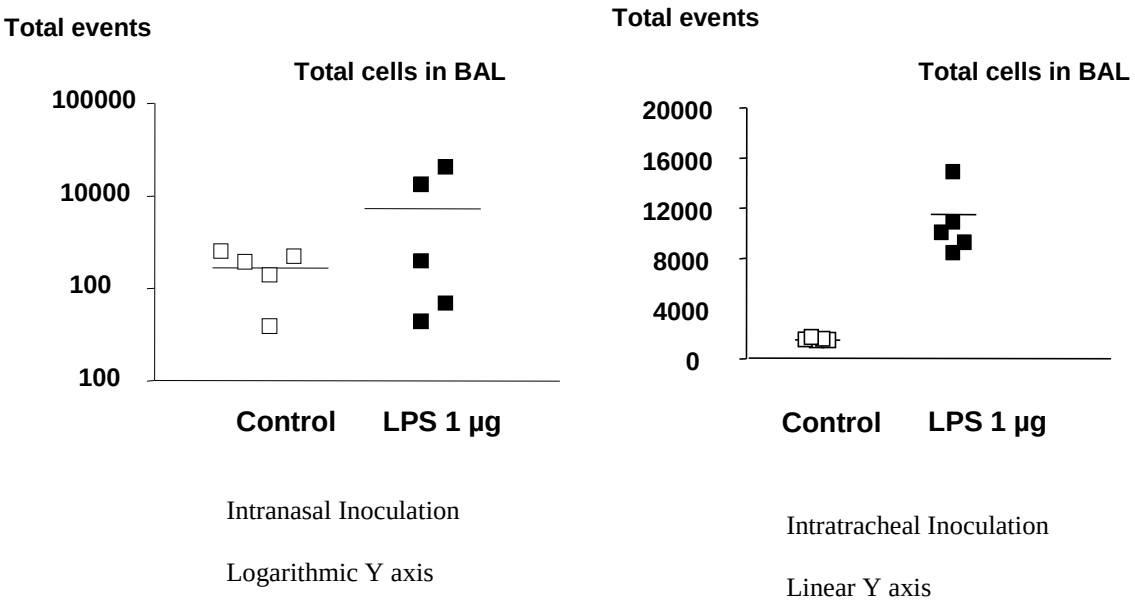
The validation test was carried out administering four groups of animals (n=5) 40 µl of LPS and saline solutions by both intranasal and intratracheal routes. Cells obtained from BAL were analyzed by flow cytometer. Results are shown in Graph 1. In it the homogeneity of the intratracheal technique cell count results is clearly better than those from the intranasal group, which vary from no effect at all to different levels of result. Notice that the two experimental groups have not the same y-axis scale. Due to the huge differences found in the intranasal group, measurements are plotted in log scale. This validation supported the use of LPS intratracheal administration as a model of lung lower airways inflammation to evaluate anti-inflammatory compounds.

The technique was then used in setting up an acute *Mycobacterium tuberculosis* infection model for therapeutic efficacy studies, fitting as the standard infection technique. Results of four consecutive control groups, (n=5) of the TB Therapeutic Efficacy Group studies are shown in Table 1. Groups (n = 5 mice/group) of immunocompetent 8-10 week old B6 female mice (Harlan, Barcelona, Spain) were infected with 10^5 CFU/mouse (target bacterial burdens at the end of the in vivo assay of 10^7 CFU/lungs). To perform infections, a vial of M. tuberculosis H37Rv Pasteur suspended in PBS-glycerol 5% freezing medium was thawed, diluted in sterile PBS to $2x10^6$ CFU/ml and instilled intratracheally (50 µL/mouse) using this non-surgical procedure. At day 9, the lungs were aseptically removed and homogenized in 1 ml of sterile-filtered water (Sigma, MO, USA) with disposable homogenizers (Covidien Ltd., MA, USA). Glycerol was added to homogenates (5% vol/vol final concentration) and they were aliquoted in two vials per mouse and frozen at -80° until plating. The number of CFU/lungs was determined by plating serial dilutions of homogenates in Middlebrook 7H11 broth containing 10% oleic acid-albumin-dextrose-catalase (BBL Middlebrook OADC, Becton Dickinson, NJ, USA). The plates were cultured for 2 weeks at 37ºC in ambient air and the colonies were counted using an automatic colony counter (aCOLyte-supercount, Synbiosis, Sinoptics Ltd. Cambridge, United Kingdom).

The figures systematically obtained in all studies demonstrate the robustness of the technique.

Four control groups of female C57BL/6 mice infected by non-surgical intratracheal instillation of 10^5 viable Colony Forming Units (CFU) of M. tuberculosis H37Rv (n = 5/group). Mice were sacrificed at day 9 after infection, lungs were aseptically obtained and, after homogenization, serial dilutions of homogenates were plated to measure the CFU burden per whole organ. Figures are CFU logarithms.

Method Validation



Graph 1: LPS inoculation technique comparative performance

Table 1: TB Therapeutic Efficacy Group studies

	Control 1	Control 2	Control 3	Control 4	Control 5	Media	StdDev
TE0362	7,31	7,27	7,16	7,36	6,87	7,19	0,20
TE0370	7,12	7,18	7,01	7,23	6,94	7,09	0,12
TE0383	7,09	7,12	7,01	7,33	7,32	7,17	0,15
TE0384	7,35	7,34	7,43	7,35	7,27	7,35	0,06

DISCUSSION

Any of the intratracheal intubation techniques described in the scientific literature (Costa *et al.*, 1986), provide a reliable way to deliver the exact amount of inocula in the right place. These techniques are also useful in models that need intratracheal delivery of compounds. In the mouse there have been described several ways to achieve this. Most of them include the utilization of tools and devices specially designed or adapted for mice (Costa *et al.*, 1986; Ho *et al*., 1973; Su *et al*., 2004; Spoelstra *et al.*, 2007).

We have been using the described technique in several different models having shown that it is as quick to perform as the intranasal administration and it is as good in minimizing suffering. At the same time it is as quantitative as the transtracheal method. Intranasal inoculation proved to be erratic in the experiments tested because the administered amount of substance or inocula that reaches the lung is variable and unpredictable, whereas transtracheal technique implied a surgical procedure, with a longer anesthesia protocol, hypothermia control and some days needed for total recovery and wound healing. In both surgical and non surgical intratracheal techniques the delivery in lower airways is granted, and also results demonstrated no reproducibility problems and good correlation between inocula or compound dosing and responses.

The aerosol generators are not always appropriate because of the microbes' characteristics or because they may alter certain molecules, in both cases preliminary studies must be carried out, as reproducibility is very difficult. In addition, depending on the generator model, and microorganisms or substances utilized, biosafety issues may arise.

The described technique allows animals to keep spontaneous breathing, but also gives the chance to ventilate if required to recover from apnea in the few cases reported, uses an ultra-short-acting anesthesia protocol that guarantees that the animals will show normal behavior, i.e. well being, in a few minutes, and also guarantees the researcher that the intratracheal access has been achieved.

As a conclusion, this technique does take about 40 seconds to perform in a highly affordable, robust and reproducible way. No mortalities were experienced, nor were lesions like edema, bleeding or laryngeal damage reported in more than ten years.

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