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# African Journal of Microbiology Research

## Table of Contents: Volume 12 Number 6 14 February, 2018

## ARTICLES

**Parameter optimization of pyoverdine content and growth kinetics on Pseudomonas fluorescens pf-10 strain in iron deficient liquid state media**
Berdja R., Abderrahmane O. and Benchabane M.

**Molecular epidemiology of Coxiella burnetii in human, animals and ticks in Bangladesh**
Md. Siddiquur Rahman, Amitavo Chakrabartty, Roma Rani Sarker, Sayra Tasnin Sharmy, Abu Sayeed Sarker, Sonia Parvin, Heinrich Neubauer and Klaus Henning

**Evaluation of safety and immunogenicity of combined blackleg and hemorrhagic septicemia vaccine**
Hunderra Sori, Shiferaw Jemberie, Martha Yami, Jalata Shuka, Birhanu Abebe and Kenaw Birhanu

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter optimization of pyoverdine content and growth kinetics on Pseudomonas fluorescens pf-10 strain in iron deficient liquid state media</td>
<td>127</td>
</tr>
<tr>
<td>Molecular epidemiology of Coxiella burnetii in human, animals and ticks in Bangladesh</td>
<td>136</td>
</tr>
<tr>
<td>Evaluation of safety and immunogenicity of combined blackleg and hemorrhagic septicemia vaccine</td>
<td>141</td>
</tr>
</tbody>
</table>
Parameter optimization of pyoverdine content and growth kinetics on *Pseudomonas fluorescens* pf-10 strain in iron deficient liquid state media

Berdja R.1,2*, Abderrahmane O.1,3 and Benchabane M.1

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Iron nutrition in bacteria presents a fundamental challenge due to its extremely low solubility in aerobic environments at moderate pH. For optimal growth, *Pseudomonas* bacteria necessitate about 10 e-6 to 10 e-7 M of bioavailable iron and therefore solve an iron supply problem for survival by synthesizing and exporting a low-molecular-weight compound called siderophore. The latter has a high iron binding capability from the bacterial cell, and then importing it once bound to iron. A system of nonlinear ordinary differential equations has been studied and a parameter identification study conducted on unknown parameters along with a sensitivity analysis to determine key factors that contribute most to the variation in model outputs (experimental observations). The model has been quantitatively validated against population size count and pyoverdine content measurements obtained from *Pseudomonas fluorescens* pf-10 strain, the model solution can be used as an indirect experimental tool for developing practical criteria in plant growth-promoting rhizobacteria (PGPR) selections which are known to provide effective rhizosphere colonization, together with accurate predictions of iron depletion in the system over time.

**Key words:** *P. fluorescens* Pf-10, pyoverdine, Iron chelating, model calibration, sensitivity analysis.

INTRODUCTION

Iron availability is low in many soils; hence, microorganisms have evolved mechanisms to acquire this nutrient by, altering the chemical conditions that affect its solubility. Microorganisms respond to Fe deficiency by production and release of specific chelators. In the rhizosphere, the demand for Fe results in competition between plants and microorganisms with the latter being more competitive due to their ability to decompose plant-derived chelators and their proximity to the root surface (Marschner et al., 2011). These chelates, which were designated earlier as siderochromes, sideramines, and sideromycins, are now conveniently

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termed siderophores, technically defined as the ferric iron specific, low molecular weight (<1500) compounds, which solubilize and transport iron to the cell.

Among siderophores, pyoverdine is a water-soluble yellow-green fluorescent (under UV light) pigment which is composed of approximately 40 different structures established from various strains. Besides that, more than 70 different pyoverdines structures are expected from isoelectric focusing and iron uptake patterns determination studies (Emery, 1977; Meyer, 2000).

Pseudomonas strains which are known to be natural siderophore producers, have been isolated from different habitats around the world. Most species are saprophytes that are commonly found in water, soil and plant surfaces (Haas and Défago, 2005; Lugtenberg et al., 2001). Among Pseudomonads, 23 species are pathogenic to plants, including Pseudomonas syringae with 36 pathovars affecting different plants. In addition, 16 species are associated with diseases in humans and animals (Peix et al., 2009).

Plant-beneficial pseudomonads also called plant growth-promoting rhizobacteria (PGPR) release a remarkable diversity of exoproducts with antimicrobial, metal chelating, lytic, and phyto hormonal activity, and some of them have a determinative role in disease suppression and plant growth stimulation by increasing the availability and uptake of mineral nutrients (Egamberdieva et al., 2011; Glick, 2012; Kami洛va et al., 2005). Indeed, diverse studies suggested that, these molecules can be bacterial signals triggering Induced Systemic Resistance (Leeman et al., 1996; Meziane et al., 2005). This is an essential prerequisite for bio control efficacy of fluorescent pseudomonads, depending on traits that allow effective root colonization and competition for nutrients and niches (CNN), that do not involve antibiotic production. It has an additional advantage that does not raise because of resistance development. Therefore, some Pseudomonas fluorescens can be considered to be safer and more practical than biocontrol strains which exhibit direct antagonistic activities against phytopathogens (Chen and Ljung, 2013; Cho et al., 2015; Lugtenberg et al., 2001).

The selection of highly competent pseudomonads requires expensive field sampling and fastidious laboratory analysis, thus predictive modelling using different type of mathematical models is an alternative way to describe and explain complex systems dynamics, which are known to be in practical multidimensional search problems with a number of local optima, which donot always give a satisfactory results (Weuster-Botz, 2000). Parameter optimisation techniques allow fair and objective assignment of parameter values so that, any differences in model performance can be attributed to differences in model structure, rather than to the relevance of the parameter values (Friedrichs et al., 2007). Parameter values assignment in biological modelling can strongly affect model performance, which can be hard to define accurately and precisely. In spite of that, a situation parameterisation is often used, according to the results of laboratory based studies (Pahlow and Oschlies, 2009).

The model calibration of the differential equation–based model has been applied to estimate unknown parameters and model predictions which have been quantitatively validated against experimental data and predict the time variations of substrate concentrations(iron) for which we have no experimental measurements to compare with. In addition, a sensitivity analysis in the vicinity of the resulting optimal parameter set was used to determine the strongest parameters that induce the largest changes in model's state variables.

MATERIALS AND METHODS

Bacteria strain and culture conditions

P. fluorescens Pf-10, an autochthonous strain isolated from palm date rhizosphere in south Algeria was used in this study (Benchabane, 2005; Toua et al., 2013). The strain was characterized to be resistant to rifampicine and positive for phosphate solubilisation, for the production of indole-3-acetic acid, siderophore, 1-aminoacyclopropane-1-carboxylate deaminase, and 2,4 diacetylphloroglucinol (DAPG), which makes it a potential PGPR (Toua et al., 2013). The bacterial culture was maintained as 50% glycerol stocks at −20°C in King's-B medium (King et al., 1948).

Bacterial cultures were grown for 40 h at 25°C, with shaking (200 rpm) in 500 ml Erlenmeyer flasks containing 125 ml King B medium (pH 7). To remove traces of iron, glassware was cleaned with 6 M HCl and with double distilled water. For growth and pyoverdine measurements, 1.4 ml samples were taken at time zero, and then at 1 h intervals over all incubation periods.

Measurement of growth and siderophore assay

Bacterial growth was estimated turbidimetrically at 600 nm. For estimation of dry cell weight, 10 mL sample was passed through a 0.2 µm filter paper, and the residue was dried overnight to a constant weight under vacuum at 70°C. The amount of siderophore secreted into the culture medium was determined by removing bacteria by centrifugation and measuring the absorbance of the supernatant at 400 nm.

Since the siderophore produced by Pf-10 strain tested positive for hydroxamate type following the method of Snow (1954), the values of extinction coefficient ε = 16,500M−1 cm−1 and of molecular weight MW=1500Da were used. The siderophore concentration (g/L) was calculated using the expression (O.D) 400 nm ×MW/ε (Meyer and Abdallah, 1978).

Presentation of the mathematical model

The model used in the present study is derived from the well-known Baranyi’s model (Baranyi and Roberts, 1994) which is widely used for its large applicability under different dynamic environmental conditions, and for the biological interpretability of its constitutive parameters (Van et al., 2005). The lag phase model was taken and consists of a non-autonomous system of four nonlinear ordinary
differential equations. The underlying assumption is that, only a fraction ($\alpha$) of the whole bacterial population ($N$) contributes to the growth process when introduced into a new environment, while the remaining cells adapt their physiological state to the new conditions (Fgaier et al., 2008).

The bacterial population is characterized by two variables, population size ($N$) and its lumped physiological state, expressed in terms of a function $\alpha(t)$. The chelator pyoverdine is described by its density ($P$). Bacterial growth depends on iron bioavailability in the system and is represented by two forms knowing: freely dissolved denoted by S, binded by chelator molecules, denoted by Q.

Model calibration

In order to get a good fit between the approximated model solution and assimilated data, the model calibration problem is formulated as well-known nonlinear least-squares (NLS) optimization problem, Where the objective function is given as below:

$$
\min_{\theta} J(\theta) = \min_{\theta} w \frac{1}{n_N} \sum_{i=1}^{n_N} \left( \bar{N}_i - N(t_i^f; \theta) \right)^2 + (1 - w) \frac{1}{n_P} \sum_{i=1}^{n_P} \left( \bar{P}_i - P(t_i^f; \theta) \right)^2
$$

$w$, is a fixed constant value equals to 0.5.

Note that, the evaluation of the objective function $J(\theta)$ requires the numerical solution of the underlying model. Thus, in order to solve the scalar least-square problem (1), sequential approach in Matlab 12 are used, by solving the optimization problem (1) through a sequential quadratic programming (SQP) technique, using a stiff solver for the underlying differential equation. A linear interpolation at specific query points is performed, in order to supply more entries for experimental data, which was found to be dense at the initial growth phase and sparse for larger time period.

To approximate realistic scenarios of microbial growth, initial guesses for the dependent state variables and model pathway parameters were provided by, generation of random parameter sets ($\theta$) using a Latin hyper cube sampling method (LHSDESIGN Matlab function) that generated 10,000 random parameter vectors in which individual parameters were sampled independently from their respective intervals. Each simulation covered a 2-day time interval after inoculation.

From the resulting calibrated model, a measure of goodness of fit for the nonlinear regression coefficient was carried out along with a post-analysis of the calibrated models thus, adjustment level and confidence regions of the model predictions is computed, together with the scatter plots of errors for $N$ and $P$.

Local sensitivity analysis

To investigate the effect of each parameter on the system behaviour, logarithmic (i.e., relative) local sensitivities, $S_{ij} = \partial \log X_i(t)/\partial \log p_j$ at time moments $t$ was calculated, according to the standard definition of Mitrophanov et al. (2007).

$$
S_{ij} = \partial \log X_i(t)/\partial \log p_j = \left( \frac{dX_i}{X_i} / \frac{dp_j}{p_j} \right)
$$

Where $X_i(t)$ is the model’s $i$th variable and $p_j$ is the model’s $j$th parameter (of the model’s 8 main parameters). By definition, each of these sensitivities reflects the magnitude of the relative change in a model’s output variable induced by a local (i.e., small) relative change in the vicinity of the optimal parameter set $\theta^*$.

To obtain numeric approximations of the derivatives in Equation 2, each parameter was individually perturbed by $\pm(10$ and $20\%)$ of its nominal value, and the derivative was approximated using the second-order central finite difference formula.

In all analyses, local sensitivities for each of the 41 evenly spaced time was calculated which discretize the total of 2 day simulation interval into 1h subintervals (that is $0\,1\,h\,etc$). To compare and rank sensitivities, absolute values were used.

RESULTS AND DISCUSSION

Parameter estimation and predictions of the mathematical model

Parameter optimisation of non-linear systems is rarely a simple task, but it is theoretically possible to objectively and fairly assign optimal to model parameters on the basis of observations, so that models can be compared in terms of structure alone, by using formal optimisation techniques (Friedrichs et al., 2007). In the present work, the parameterisation of the governing system of differential equations is set according to real data entering results obtained from laboratory based study on bacterial growth and pyoverdine production, the integration of the system was performed for a sufficiently long time period to reach the growth limiting plateau; no stability problems have been noticed.

However, model solution as well as the values of the objective function, have been found to be sensitive to the value of the free factor ($\omega$). Reliable solutions were obtained in our simulation experiments as, a solution of the scalarized vector optimization problem $\theta$, which was set up to be a balanced design model, and did not give advantage to biomass production termat the expense of pyoverdine content (Table 1). Conversely, with the current design, the model solution is in favour of biomass production giving rise to goodness of fit which is always superior to that of pyoverdine contents.

Experimentally measured data were successfully captured over the entire period with model fit, the quality of agreement between experimental and predicted results provide confidence in the assumptions and mechanisms in which the mathematical model is built as we correspondingly get a goodness of fit of 0.94 and 0.84 (Figure 1A and B) for biomass ($N$) and Pyoverdine ($P$). To get a visual representation of the quality of fit, model solutions along with experimental data were shown.

For pyoverdine ($P$), quantitative fit was also good for much data although, some discrepancies occurred from the onset stage of the exponential growth phase, even if we’ve initially performed an interpolation to supply more data points to the assimilated data. This lack of fit can be explained by the fact that observations are currently insufficient to accurately constrain the number of parameters required by even the simplest biological models, which share a common typical large variability due to randomness and underdetermination inherent to
Table 1. Initial, optimal and parameter range variation vectors associated with biomass and pyoverdine content.

<table>
<thead>
<tr>
<th>Parameters symbols</th>
<th>Initial values</th>
<th>Optimal values</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (µM)</td>
<td>1.604</td>
<td>2.3326</td>
<td>[0 - 4]</td>
</tr>
<tr>
<td>δ (µM)</td>
<td>0.127</td>
<td>0.0412</td>
<td>[0 – 0.13]</td>
</tr>
<tr>
<td>B (h⁻¹/OD)</td>
<td>0.857</td>
<td>0.4109</td>
<td>[0 – 1]</td>
</tr>
<tr>
<td>σ (h⁻¹µM)</td>
<td>0.089</td>
<td>0.0534</td>
<td>[0 – 0.9]</td>
</tr>
<tr>
<td>Yn (µg µl⁻¹)</td>
<td>0.329</td>
<td>0.4952</td>
<td>[0 – 1]</td>
</tr>
<tr>
<td>μ (h⁻¹)</td>
<td>0.122</td>
<td>0.1115</td>
<td>[0 – 0.5]</td>
</tr>
<tr>
<td>S∞ (µM)</td>
<td>0.609</td>
<td>0.6857</td>
<td>[0 – 0.5]</td>
</tr>
<tr>
<td>σ (µM)</td>
<td>0</td>
<td>0.0617</td>
<td></td>
</tr>
</tbody>
</table>

State variables

<table>
<thead>
<tr>
<th></th>
<th>Estimated variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (OD)</td>
<td>0.05</td>
</tr>
<tr>
<td>S (µM)</td>
<td>Log₁₀(100)</td>
</tr>
<tr>
<td>P (OD)</td>
<td>0.001</td>
</tr>
<tr>
<td>Q (µM)</td>
<td>0</td>
</tr>
</tbody>
</table>

Legend: Yn(µg µl⁻¹), growth yield constants, commonly referred to as the substrate-to-biomass yield factor, used to convert between cell growth rate and substrate utilization; μ (h⁻1), specific growth rate as a function of substrate concentration; K (µM), is the value of the concentration of nutrients S where the specific growth rate μS has half its maximum value (half-growth concentration rate); S∞ (µM), iron concentration triggering pyoverdine synthesis; σ (h⁻¹µM), coefficient related to iron chelation rate; v(Unitless), recovery rate of PGPR population; both δ (µM) and B (h⁻¹/OD) are coefficients related to linear functions dependent on the amount of freely available iron in the system; α(t), function of physiological adaptation state given by d fun.

Figure 1. (A) Quality of the fit between experimental vs. predicted growth rate. (B) Quality of the fit between experimental vs. pyoverdine content.

A $R^2 = 0.94$

B $R^2 = 0.84$

Inverse modelling problems, where the uniqueness of estimated coefficients is not guaranteed by any optimal point solution ($θ^*$).

In addition, the lack of fit may occur because of uncertainties related to variables that cannot be measured, missing data, physical forcing and initial conditions or plainly due to lack of knowledge (Banga and Balsa, 2008; Friedrichs et al., 2007, Kravaris et al., 2013). As a consequence, several parameters could be set anywhere across a wide range of values while providing a similarly good fit to the assimilated data (Groetsch and Groetsch, 1993). Last, this lack of fit may have been due...
to experimental variability which would require more sensitive and precise experimental systems and techniques or some structural deficiencies in the underlying model assumptions, as it does not account for:

i. Substrate consumption for maintenance,
ii. Substrate breakdown in the medium,
iii. Additional substrate added during the growth process,
iv. More than one limiting substrate, and finally
v. Competition between more than one bacterial population.

In Figure 2A and B we include the corresponding predicted concentrations of freely available iron (S) and chelated iron (Q), for which we do not have measurements to compare with; together with residuals plots (Figure 2C and D) for biomass (N), and pyoverdine content (P), which shows a mean expectation of zero and non-random small residuals values of the nonlinear growth kinetic model.

Robust sampling scheme was used such as the Latin hypercube method to helped the optimisation routine process to converge reliable solutions, by generating uniformly sampled initial guesses from each respective range of bounded domain (Table 1). This prevented each parameter from taking unrealistic values falling outside the certain range as reported by parameters mean values and solution region in Table 2, which was calculated each term of the optimal parameter vector $\theta^{*}$, yielding a goodness of fit superior or equals to 0.7 over all simulations runs.

However, this approach has some disadvantages as in general; it does not allow all possible outcomes to be evaluated, such as all stationary states, periodical and chaotic regimes, or how state variables depend on the parameters (McKay et al., 1979; Schartau and Oschlies, 2003), confidence interval (CI) of the best optimal parameters vectors $\theta^{*}$.

Globally, model performance is rather good, the dimension of the solution region is consistent and the misfit costs are statistically indistinguishable from the absolute minimum, since all reaction parameters are
robust, that is no strong variations are found (Table 2). There is as an indication that, the model is not over-parameterized (model with large number of parameters) or over-fitted, i.e. the goodness of fit is due to the flexibility and model structure that indeed gives a robust description of the available data, without resorting to model recalibration, except for the freely available iron on which we’ve initially performed a log10 transformation in order to get consistent outputs results.

On the other hand, and contrary to many predictive models that based on experimental data, to impose a mathematical structure which pre-specifies a fixed maximum population density Nmax, the present predictive model can cope with any maximum population density induced by essential substrate depletion, which is able to predict the amount of pyoverdine along with the freely available and chelated iron from growth measurements and vice versa, during any desired culture growth period (Gábor and Banga, 2015; Van et al., 2005).

**Model robustness analysis**

**Parameter robustness analysis**

As the impact of model parameters on model outputs sensitivity changes over time, time-dependent parameter sensitivity analysis has been proposed to study the effect of parameter variation on model output at different time (Liu et al., 2005; Savageau, 1971). This is much more evident, since some parameter may have a positive impact on the change of a model output at some stage, and precipitously switch from positive to negative due to the complex feedbacks, associated with biological networks.

Therefore, one needs to know not only which parameters are critical for affecting model output, but also at which time do point change occur and how long it last. For this aim, the robustness of our results were tested by performing local sensitivity analysis in the vicinities of 10,000 randomly selected parameter sets.

We identified the parameters, which induced the largest variations in the concentrations of four state variables across 10,000 simulations, ran with random parameter sets. Globally, we verified that for > 70% of the simulations, the sensitivities of the two parameters (µ) and (Yn) were the most important for majority of the considered time points simulations, with respectively 39 and 34% occurrence frequency when all state variables are considered (Figure 2). Below, representative simulation results for 2 days (8, 16 and 24h...) are shown in Figure 3 aimed at each of the 10,000 randomly selected parameter sets. This is the parameter at time (8 to 16 h) after inoculation, for variable microbial biomass (N). However, this sensitivity tend to decrease over time in favor of the parameter (Yn). Conversely, for variable pyoverdine content (P), the sensitivity of the parameter (Yn) tend to increase over time to become the unique most sensitive parameter at the end of the simulation. This increase occurs during the lag-phase (1 to 18 h after inoculation) at the expense of the specific growth rate (µ) and recovery rate (ν) of P. fluorescens pf 10, when grown in a new media. Commonly, Pseudomonas stains have to adapt, grow and eventually produce sufficient amount of pyoverdine molecules to chelate the freely available iron present in the culture broth media to use it in its own metabolism (Swinnen et al., 2004).

On the other hand, variables related to substrate availability show that, the freely available iron (S), is most sensitive to parameter (µ). However, this parameter tend to decrease in occurrence frequency as time goes by to the advantage of (δ); (Yn) and (σ), in order of decreasing effect magnitude. For sensitivities, the later three aforementioned parameters represented less than 30% occurrence frequency while (k, Beta, S°) did not exceed 5% occurrence each. Finally, chelated iron (Q), is exclusively sensitive to the parameter (δ) at the earlier phase of bacterial growth (Lag-phase: 1to 8 h), this sensitivity tends to decrease over time and become null when the whole quantity of freely available iron is chelated and uptaken into the microbial cytosol after 24 h of microbial growth. This phase correspond to the increasing part of iron chelation curve shown in (Figure 2B).

The decrease of (δ) is associated with an increase in

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean value</th>
<th>CI (95 and 99%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (µM)</td>
<td>1.9380</td>
<td>[1.8320; 2.0441]</td>
</tr>
<tr>
<td>δ (µM)</td>
<td>0.0366</td>
<td>[0.0333; 0.0398]</td>
</tr>
<tr>
<td>β (h−1/OD)</td>
<td>0.5191</td>
<td>[0.4999; 0.5383]</td>
</tr>
<tr>
<td>σ (h−1/µM)</td>
<td>0.2168</td>
<td>[0.1974; 0.2361]</td>
</tr>
<tr>
<td>Yn (µg µl−1)</td>
<td>0.5774</td>
<td>[0.5626; 0.5922]</td>
</tr>
<tr>
<td>µ (h−1)</td>
<td>0.3569</td>
<td>[0.3428; 0.3709]</td>
</tr>
<tr>
<td>S° (µM)</td>
<td>0.4634</td>
<td>[0.4484; 0.4785]</td>
</tr>
<tr>
<td>ν</td>
<td>0.1921</td>
<td>[0.1821; 0.2021]</td>
</tr>
</tbody>
</table>

**Table 2. Confidence interval (CI) of the best optimal parameters vectors θ̂.**
the sensitivity of \((\mu)\) which reach its maximum, after roughly 24 h of microbial growth then decrease under the effect of \((\sigma)\) which gradually become the most sensitive parameter with higher occurrence frequency. The latter, starts from the middle stage of the exponential growth phase and last until the last stage of growth is reached (stationary phase). In parallel, this phase is associated with the decreasing phase of iron cheation curve shown in Figure 2B.

Notably, the parameters \((\mu)\) and \((v)\) induce changes that are negatively related to the parameters \((Y_n)\); \((\delta)\) and \((\sigma)\), since they all induce changes in opposite directions over time. Specifically, a decrease of \((\delta)\) is associated with an increase of \((\mu)\), and inversely, an increase of \((\sigma)\) and \((Y_n)\) is associated with a decrease in the sensitivity of \((\mu)\) and \((v)\) (Figure 3). Overall, varying model parameters in the vicinities of 10,000 randomly selected parameter sets, permitted to demonstrate that the model is far more sensitive to some parameters than the others. In particular, the parameters is associated with microbial growth \((\mu, v)\); Chelate amount \((Y_n)\) and iron availability \((\delta, \sigma)\). However, those results can vary if one change the value of the scale parameter \(w\) in the cost function, which was set to be equal to 0.5, to avoid giving advantage for microbial growth at the expense of pyoverdine synthesis rate (balanced design), over all simulations.

**Conclusion**

Dynamic modelling gives an efficient structure to comprehend process in natural system frameworks. Parameter estimation in nonlinear dynamic modelling remains an extremely difficult task when inverse problems is dealt with because of their non-convexity and ill-conditioning state related to overfitting and local solutions. In modelling studies, if the long-term behaviour of the population is of interest the lag phase period can often be ignored, as it is the situation with presumably most applications in biological modelling which focus on persistence and survival.

In the simulation study, calibrated model is tested with a new data set for cross-validations by considering the same model structure and different experimental condition which adopted a computational approach focused on simulations in order to evaluate the model’s validity and check agreement with experimental data. In addition, the robustness of the computational model is investigated by a local sensitivity analysis in the vicinity of the resulting optimal parameter set. Predictions of the model compared well with experimental data obtained from microbial growth and pyoverdine content measurements. In addition, they nicely predict iron dynamic for which we did not have data to compare with.
Preceding, sensitivity analysis allowed us to pinpoint key factors (model inputs) that contribute most to the variation of model outputs. Thus, key dynamic features of the biomass and pyoverdine production can be explained and predicted using a parsimonious computational modelling approach.

However, on the other side one should be cautious since the numerical solution assumes that all parameters are independent random variables. Thus, further studies should consider the analysis of the error covariance matrix for the optimal parameters, in order to determine (i) poorly-constrained parameters (ii) correlated and partially correlated parameters (iii) how under-determination is handled by optimisation approach (vi) the exact number of parameters that could be constrained by the available data and (vii) a sensitivity analysis handling the interactions amongst more than one parameters.

Finally, the presented model approach could be useful not only as an indirect experimental tool for evaluating empirical data-based theories, but also as the basis for microbial biomass and secondary metabolite prediction which would reduce or eliminate the need for expensive laboratory analysis procedures, and help for the design and optimization of operation of large-scale production of microbial inocula and developing practical criteria in making PGPR selections, which are known to provide effective rhizosphere colonization. In perspective, further studies should focus on biofilm modelling since the interaction of pseudomonads population which are known to be natural biofilm formers and resource dynamics can be different from those occurring in suspended state media.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES


Full Length Research Paper

Molecular epidemiology of *Coxiella burnetii* in human, animals and ticks in Bangladesh

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Q fever, caused by *Coxiella burnetii* is a well recognised zoonotic disease around the world which is endemic in many countries. In Bangladesh, a very few information is available in men and animals about this disease. Thus, the objective of this study is to determine the presence of Q fever in animal (goat and cattle) and human population including the tick which is a vector of the disease. A total of 172 animals with abortion and reproductive disorder history were collected from Shahjadpur Upazilla of Sirajgonj district in Bangladesh. 150 sera sample from patients of Mymensingh Medical College Hospital were collected which had a history of pyrexia. 127 tick samples were collected from the body of animal randomly. The samples were tested with CHEKIT Q-Fever antibody Enzyme Linked Immunosorbent Assay (ELISA) Test Kit (indirect ELISA) primarily and the positive sera were retested by real time polymerase chain reaction (PCR) to detect DNA of *C. burnetii*. Indirect ELISA revealed the prevalence result of 6.97 and 0% in animals and humans, respectively and only one tick was Reverse transcription Polymerase Chain Reaction (RT-PCR) positive (0.79%). It is thus observed that this is the first time the presence of *C. burnetii* in ticks using real time PCR is reported.

Key words: *Coxiella burnetii*, Enzyme Linked Immunosorbent Assay (ELISA) antibody, polymerase chain reaction (PCR), sera samples.

INTRODUCTION

Q fever is a disease which is zoonotic in nature caused by *Coxiella burnetii*. Cattle, sheep and goat act as a
primary reservoir host of this disease (Arricau-Bouvery and Rodolakis, 2005; McCaughey et al., 2011) and has also been found in other mammals like birds, fish, reptiles, ticks and other arthropods (Porter et al., 2011). The personnel related with livestock (farmers, veterinarians, abattoir workers, laboratory personnel working with Coxiella burnetii) are at higher risk for Q fever (Maurin and Raoult, 1999). Wild and domestic mammals serve as reservoirs and are part of the bacterium’s life cycle. The environment is contaminated by vaginal secretions, placenta, milk, feces, urine, saliva and other by-products of infected animals (Porter et al., 2011). C. burnetii is transmitted mainly by inhalation or by contact with infectious tissues, although tick-transmission of this bacterium has also been reported (Rolain et al., 2005). Human infections with C. burnetii are usually transmitted by aerosol during contact with infected animal feces or bird residues (Tissot-Dupont et al., 2004). Abortion, infertility and other reproductive problems are the main problems in animal due to Q fever (To et al., 1998). Therefore, significant economic losses occurred when a farm is infected with Q fever (Porter et al., 2011). In humans, it affects the respiratory system, musculoskeletal system, digestive system, nervous system and cardiovascular system (Karakousis et al., 2006).

The disease has been well documented and reported in many countries of the world (Porter et al., 2011; McCaughey et al., 2011; Arricau-Bouvery and Rodolakis, 2005; Maurin and Raoult, 1999). Haider et al. (2015) and Rahman et al. (2016) reported the presence of Q fever in Bangladesh. In Bangladesh, animal and human density is quite high (World Bank, 2007) and they mostly share the same premises which facilitates the transmission of Q fever and other emerging zoonotic diseases from animals to humans and vice versa. Rahman et al. (2016) reported the prevalence of Q-fever in domestic ruminants by serological tests and immunoglobulin G of C. burnetii was detected by Haider et al. (2015) with a prevalence of 0.7% (8/1149) in the ruminants, specifically 0.65% (4/620) in cattle, and 0.76% (4/529) in goats by using enzyme linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA).

The limitations of the studies on Q fever in Bangladesh cited above are; this study used only a small number of samples from abortion positive herds and others only from sick animals admitted in the hospitals. Serological tests only, were used without credible information about the sensitivity and specificity of the tests. Moreover, there is no information regarding the status of C. burnetii in humans and ticks. A systemic scientific study using molecular technique is essential for characterization of C. burnetii in Bangladesh.

**MATERIALS AND METHODS**

The study was carried out at the Zoonotic Disease Diagnostic Laboratory, Medicine Department, Faculty of Veterinary Science, BAU, Mymensingh, Bangladesh with the collaboration of OIE Reference Laboratory for Q fever, Institute of Bacterial Infections and Zoonoses, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institute, Naumberger Str. 96a, 07743 Jena, Germany. The study was done through the following steps below;

**Collection of samples**

A total of 172 sera samples of animals (goat and cattle) were collected which had the history of reproductive problem such as anestrous, abortion, retention of placenta etc. from Shahjadpur Upazilla of Sirajgonj district in Bangladesh. 5ml of blood were collected from each animal with a10 ml sterile syringe. The serum was made by keeping it undisturbed for about 10 h. The supernatant was collected and centrifuged. Each serum was collected separately in a sterile screw capped tube using micropipette and tube was labeled.

Blood sample from 150 patients with the history of pyrexia of unknown origin was performed with the help of physicians from Mymensingh Medical College Hospital and each serum was collected and stored at -20°C until further use. Sampling were performed with respect to human consent and animal welfare.

A total of 127 ticks were examined carefully by hand picking in other to keep the mouth part intact. Collected ticks were kept in vials with necessary examination and identification done in the laboratory according to the standard method, following the keys and description given by Soulsby (1962).

**Serologic surveillance of the diseases**

All reagents were mixed by gentle shaking at 18 to 26°C. Samples were checked twice and their optical densities (OD) were averaged. The OD of negative control was corrected by subtracting it. CHEKIT Q-Fever antibody ELISA Test Kit (IDEXX, Liebefeld-Bern, Switzerland) was used to test the sera (Gwida et al., 2014).

**DNA extraction and real time polymerase chain reaction (PCR)**

The tick was crushed with mortar and pestle and DNA was extracted (Schmoock et al., 2014). High pure polymerase chain reaction (PCR) Template Preparation Kit™ (Roche Diagnostics, Mannheim, Germany) was used according to the instructions of the manufacturer. With a Taq Man based real-time PCR, assay was performed for DNA quantification targeting the transposase element (Klee et al., 2006).

The real-time PCR assay was performed with the primers and the conditions as described in Chakrabarti et al. (2016). Samples were considered positive with a cycle threshold (Ct) < 40 (Boarbi et al., 2014).

**Statistical analysis**

Data were entered into MS excel 2007. Cleaning and processing of data were performed then transferred to Specific Statistical software
Table 1. Prevalences of *C. burnetii* in animals, humans and ticks.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tested</th>
<th>Positive ELISA</th>
<th>Prevalence (%)</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>172</td>
<td>12</td>
<td>6.97</td>
<td>Negative</td>
</tr>
<tr>
<td>Human</td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Tick</td>
<td>127</td>
<td>ND</td>
<td>0.79</td>
<td>Positive</td>
</tr>
</tbody>
</table>

ND, Not done.

**RESULTS AND DISCUSSION**

The overall seropositivity of *C. burnetii* antibodies were 6.97% in animal and 0% in humans and no positive seropositive sera was found by real time PCR (Table 1).

*C. burnetii* was detected in only one tick (0.79%) through real time PCR (Figure 1). In this study, it was observed that seroprevalences of *C. burnetii* antibodies were higher in animal which is same as other authors (Khalili and Sakhaee, 2009; McQuiston and Childs, 2002). According to Rahman et al. (2016) in Bangladesh, the prevalence of the disease in ruminant was 6.38% also, by Haider et al. (2015) the prevalence of the disease was 0.7% (8/1149) in ruminants (0.65% in cattle and 0.76% in goats). There were no positive results in humans using indirect ELISA in this study and one tick was found real time PCR positive.

Normally, the diagnosis of Q fever is done by serological test, culture of the organism and conventional polymerase chain reaction (PCR). The pathogen isolation is the best method for proper diagnosis, but working with this organism is hazardous for laboratory workers and thus, level 3 biosafety cabinets are required for it (Arricau-Bouvery and Rodolakis, 2005; Fournier et al., 1998). Epidemiological investigations mainly rely on serological tools due to the lack of cardinal signs of the disease. Therefore, ELISA was chosen to detect Q fever seroprevalence in man and animals for its cheap rate and safety level (Rousset et al., 2010).

Samples from these areas where low or subclinical coxiellosis present only serological test may not be specific and sensitive enough for the diagnosis of *C. burnetii* infection due to cross-reactions (Rousset et al., 2009). Moreover, seroconversion occurs after 3 to 4 weeks post infection in time frame which is a limitation of
serological diagnostic methods. In addition, antibodies against *C. burnetii* often appears late in the course of the disease, therefore it is difficult to diagnose in early stage only by serological test in animals. Although, early diagnosis is necessary to treat the affected one. DNA-based method is the best method for early diagnosis. Methods such as PCR, nested PCR and real-time PCR have successfully been used for clinical identification of *C. burnetii* (Kato et al., 1988; Berri et al., 2000; Klee et al., 2006).

In this study, there were no seropositive cases in humans and none of these seropositive samples of the animal was positive in real time PCR. Other authors had also reported positive value in ELISA tested sera sample but all real time PCR were negative (Khaled et al., 2012). Due to large population of human (above 16 crores) and animal in Bangladesh (23.4 million cattle, 1.86 million buffaloes, 33.5 million goats, and 1.1 million sheep), the zoonotic diseases spread very fast. Since Q fever is a zoonotic disease and social living pattern of Bangladesh which allows intermixing of species, it can be an outbreak any time anywhere.

**Conclusion**

This study thus proves the prevalence of Q fever in animals in Bangladesh which is observably the first time the presence of *C. burnetii* in ticks using real time PCR is reported. Further research should be conducted by collecting the animal and human sample from the same geographical area for better understanding of the transmission of disease between them and actual prevalence on that area.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**ACKNOWLEDGEMENTS**

Authors are thankful to Ministry of Science and Technology, Government of Peoples Republic of Bangladesh and DAAD (Deutscher Akademischer Austausch Dienst-German Academic Exchange Service), Bonn, Germany for financial support and to first author and Director, Mymensining Medical College Hospital for their cordial cooperation in this study.

**REFERENCES**


Evaluation of safety and immunogenicity of combined blackleg and hemorrhagic septicemia vaccine

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National Veterinary Institute, P. O. Box 19, Bishoftu, Ethiopia.

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To evaluate the safety and immunogenicity of combined hemorrhagic septicemia (HS) and blackleg (BL) vaccine, combined vaccine was produced in two different formulations (group one, G1 and group two, G2) and compared with safety and immunogenicity of monovalent HS and BL vaccines in different doses. G1 combined vaccine was vaccinated in 4 and 2 ml in to five calves each while G2 combined vaccine, the monovalent HS and blackleg vaccines were vaccinated in 2 and 1 ml amount into to five calves each leaving ten non-vaccinated calves managed similarly as vaccinated ones. The safety test was made by giving single and double doses of experimental vaccines and double doses of monovalent vaccines in each case using two calves per dose group. The immunogenicity of BL vaccine component in the combined vaccine was evaluated by using 10 guinea pigs per group for six different doses of combined and monovalent blackleg vaccine, leaving 10 non-vaccinated controls. The study indicated that the protection against virulent challenges for animals vaccinated with G1 combined vaccine at 2 ml, G2 vaccine at 1 ml, monovalent HS and BL vaccine vaccinated at 1 ml doses was by far less than 90%. On the other hand, protection against experimental challenge for G1 vaccine vaccinated in 4 ml amount was 100% against both HS and blackleg virulent challenges while the protection against experimental challenge for G2 vaccine vaccinated in 2 ml amount was 66.67% against the HS virulent challenge and 90% against BL virulent challenge. So G1 combined vaccine vaccinated in 4 ml was found to be the best candidate vaccine according to this experiment which needs to be confirmed at field test before use for mass vaccination.

Key words: Combined, hemorrhagic septicemia, blackleg, safety, immunogenicity.

INTRODUCTION

Veterinary vaccines have been known to be the most cost-effective tools for the prevention and control of infectious disease. Their uses have an enormous impact on disease both in eradication of the disease totally, as was seen in Rinder pest, and reducing the incident of disease occurrence, both meant to benefit the animal owners and the animal itself specifically and further the whole community and the country in general. There are multiples of diseases for which vaccines have been developed and vaccination is used to prevent and

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control both the incidence and impact of those diseases. However in developing countries like Ethiopia the vaccination coverage is not comparable with necessity and disease prevalence (personal observation). Among factors that reduce vaccination coverage, the budget (logistics) issue is mentioned as the primary one limiting the delivery of vaccines as a control means (to contain the outbreak) only (Stevenson, 2009).

Even though the government has to plan and implement mandatory vaccinations for animal disease prevention, other strategies which support the reduction of vaccination costs and facilitate the vaccination schedule are also necessary. One important approach is the use of combined vaccines (Stämpfli, 2016; Johns and Hutter, 2010; Dodd, 2003; Edwards, 1994) whose uses have the enormous advantages as it provides the ability to administer two or more antigens in a single shot, thus decreasing the discomfort for vaccine recipients and owners by reducing number of injections, reducing the cost of the vaccine delivery process as it reduces number of contacts, increasing vaccination coverage for the vaccines concerned as it could minimize chances of dropouts and also saves budget to cover other sites, and aiding to make the vaccination schedule safe that in turn minimizes the frequency of animal gathering and thereby decrease chance of disease transmission (Thrushfield, 1995).

A combined vaccine has been known to be prepared by mixing two or more live or inactivated or purified antigens at one of the end production processes or by mixing two vaccines at the time of application of the vaccines to prevent multiple diseases or to prevent one disease caused by different strains or serotypes of the same organism (Pastoret et al., 1997).

Epidemiologically it has been known that hemorrhagic septicemia occurs following stressing conditions especially at the beginning of rainy seasons when animals are at poor body condition and throughout the rainy season if animals are at poor body condition as this season is conducive for the transmission of pasteurella organisms (Radostits et al., 2007). On the other hand even if blackleg has been known to occur throughout the year, it is worse in spring and in fall. This indicates that disease prevention activities for both diseases. Blackleg and hemorrhagic septicemia could be synchronized and vaccination against both diseases could be made together. In Ethiopia despite the presence of constant threat both by blackleg and hemorrhagic septicemia, the vaccine comprising both antigens in single preparation is absent even if important. A report by Jabari et al. (2008) indicated that there is evidence of production of potent combined blackleg and hemorrhagic septicemia vaccine in Iran. Similarly according to different researchers (Srinivasan et al., 2012; Ghanem and Ghanem, 1987), the immunity conferred by combined vaccine of blackleg and hemorrhagic septicemia was similar to that obtained by each vaccine alone. The main objective of this study is therefore to evaluate the safety and immunogenicity of combined hemorrhagic septicemia and blackleg vaccine produced under experimental batch.

MATERIALS AND METHODS

Description of the study area

This experimental study was done in National Veterinary Institute (NVI) in Bishoftu town which is situated 47 km south east of the capital city, Addis Ababa. It was found at 9°N latitude and 4°E longitudes at an altitude of 1850 m above sea level in central highlands of Ethiopia (NMSA, 1999). National veterinary Institute is the sole veterinary vaccine producing laboratory in Ethiopia and currently produces over 260 million doses of veterinary vaccines each year.

Laboratory animals management and antibody screening

52 male calves obtained from market and 70 male guinea pigs which were bought from Ethiopian Public health institute (EPHI) were used for this study. The animals were given a quarantine time of two weeks before the start of the actual experiment. During the quarantine period and thereafter during the experiment the animals were kept on management practice recommended for these animals (housing, feeding and watering). Calves were screened for P. multocida type B2 antibodies ahead of the experiment.

Experimental design

Laboratory animals grouping

Guinea pigs: 70 guinea pigs of 300 to 400 g size were used to test the immunogenicity of blackleg component.

CALVES: 40 calves aged between 6 month and 1.5 year was used for immunogenicity test while 12 calves of similar age group were used for safety test. Sera samples were taken from these calves and screened for presence of P. multocida type B2 antibody by indirect haemagglutination test according to OIE (2012). Those which were seronegatives were used.

Combined blackleg and hemorrhagic septicemia vaccine production

Clostridium chauvoei antigen preparation

18 L C. chauvoei inoculums media was inoculated with known C. chauvoei seed (local isolate) and incubated overnight after which sample was aseptically taken in class II level biological safety cabinet and checked for purity by Gram’s stain. Then this inoculum was added to sterile 400 L liver and meat broth in 500 L size capacity fermenter. 50% glucose was also added to act as a carbon source in 9 L. The whole mix is incubated at 37°C for 24 h and when it showed full growth, sample was taken and purity checked and inactivated with 37% formalin at rate of 0.7% of culture volume. Then inactivation test and purity test were made both in guinea pigs and in laboratory media according to standard operating procedure for production of blackleg vaccine (Birhanu, 2015a, b; Tsegaye,
Hemorrhagic septicemia antigen preparation

18 L Pasteurella multocida inoculum media was inoculated with known P. multocida type B:2 (local isolate used by NVI), and incubated overnight after which sample was aseptically taken in class II level biological safety cabinet and checked for purity by Gram's staining. Then this inoculum was added to sterile 400 L liver and meat broth in 500 L size capacity fermenter. Glucose (2.5%) was also added to act as a Carbone source in 9 L amount and 3 L sterile horse serum was added to aid the growth. The whole mix was incubated at 37°C for 18 to 20 h and when it showed full growth, sample was taken and checked for purity and inactivated with 37% formalin in 0.5% amount. Then inactivation test and purity test were made both in rabbits and in laboratory media (Birhanu, 2015a, b; Tsetarge, 2015a, b; OIE Manual, 2012).

Preparation of combined vaccine

Group one (G1) combined hemorrhagic septicemia and blackleg vaccine was prepared by mixing the separately mixed 9 L clostridium chauvoei antigen with 1 L 10% aluminium potassium sulphate adjuvant and separately mixed 5 L P. multocida type B:2 antigen with 4 L saline and 1 L 10% aluminium potassium sulphate adjuvant: that is in equal proportion which is dispensed in 100 ml polypropylene vials.

Group two (G2) combined vaccine was prepared by mixing nine liter clostridium chauvoei, nine liter P. multocida type B:2 antigen and 2 L of 10% aluminium potassium sulphate and dispensing the mix in 100 ml polypropylene vials.

The remaining of the bulk in separate fermenters containing C. chauvoei and P. multocida type B:2 was used to prepare monovalent blackleg and hemorrhagic septicemia vaccine respectively part of which is utilized as positive control during safety and immunogenicity test of the combined vaccine following the usual procedure of the National veterinary institute, Ethiopia (Birhanu, 2015a, b; Tsetarge, 2015a, b).

Purity and inactivation tests

The combined vaccines (G1 and G2) and monovalent vaccines were tested for freedom from any contaminant on VF media, 10% horse serum supplemented tryptose soy broth and agar incubated both aerobically and anaerobically, and saboraud agar (Birhanu, 2015a, b; Tsetarge, 2015a, b; OIE Manual, 2012; Misra, 1991).

Safety test

The combined vaccines (G1 and G2) were tested as G1 in 4 and 8 ml doses per animal; G2 in 2 and 4 ml doses per animal and the monovalent hemorrhagic septicemia and blackleg vaccine was given in 4 ml for safety test and test animals were monitored for three weeks (Birhanu, 2015a, b; Tsetarge, 2015a, b; OIE Manual, 2012 and Misra, 1991).

Immunogenicity test

All vaccine groups were subjected to sterility and safety testing before using them for immunogenicity test according to British Veterinary Pharmacopoeia (2010). Immunogenicity test was done separately for hemorrhagic septicemia and blackleg vaccine components:

Immunogenicity of hemorrhagic septicemia component: 40 calves were used for the immunogenicity test of the haemorrhagic septicemia component of the combined vaccine (10 animals each for G1 and G2), 10 animals for monovalent hemorrhagic septicemia vaccine as positive control and 10 calves as negative control (non-vaccinated). The G1 combined vaccine was vaccinated in 2 and 4 ml per dose while the G2 combined vaccine was vaccinated in 1 and 2 ml while the monovalent Hemorrhagic septicemia vaccine was vaccinated in 2 and 1 ml per animal. These animals were followed for nine months and challenged twice (between 5-6th month and at 9th month post initial vaccination) subcutaneously with 1 ml culture containing 5 x 10^7 CFU of virulent P. multocida type B: Three animals were challenged per group each time according to (Indian Pharmacopeia).

Post challenge, the calves were observed for evidence of clinical symptoms of hemorrhagic septicemia and death until 7 to 10 days. The animals’ rectal temperature was also recorded twice in the morning and in the afternoon. The body temperature was termed febrile if > 39.5°C (Radostits et al., 2007).

Immunogenicity of Blackleg component of the combined vaccine: Seventy (n=70) 300 to 400 g weighing guinea pigs were used to test the immunogenicity of the blackleg component of the combined vaccine and monovalent blackleg vaccine (G1 in 2 and 4 ml; G2 in 1 and 2 ml; monovalent blackleg in 1 and 2 ml) each group comprising 10 guinea pigs including the non-vaccinated control. According to Indian pharmacopeia (http://ipc.nic.in/super/users/writecomm1main.asp), booster dose was given after 28th days of initial vaccination subcutaneously and challenged with 2 ml of pure clostridium chauvoei after 14th day of giving booster dose. Post challenge, the guinea pigs were observed for evidence of clinical symptoms of blackleg like swelling of muscel with crepitating sound and death until 7 to 10 days (Radostits et al., 2007).

Bacterial isolation from clinical cases: Recently dead or clinically sick calves were thoroughly gross examined and samples like spleen, heart, heart blood and liver were taken and the cause of death was identified whether it was the organism used for challenge, P. multocida type B2 via proper Pasteurella multocida isolation and identification procedure (Quinn et al., 2002). Similarly from recently dead or seriously sick guinea pigs, swab sample taken from muscel and liver was cultured and culture suspension was tested by PCR to confirm whether the cause of death was C. chauvoei or not (Vijoen et al., 2005).

Statistical analysis

The complete data concerning vaccine combination and associated quality control tests (inactivation, freedom from contaminant, safety, immunogenicity and potency tests) were carefully recorded in Ms Excell spreadsheet (Microsoft office 2007 and analyzed using descriptive statistics (SPSS version 20).

RESULTS

Vaccines purity and inactivation tests

No growth of any organism was detected after inoculation of the prepared vaccines in VF or meat and liver broth media, 10% horse serum supplemented tryptose soy
Table 1. Percentage protection of calves with virulent *P. multocida* type B2 challenge between fifth and six month post vaccination.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>No. challenged</th>
<th>Vaccination volume/dose (ml)</th>
<th>Challenge dose (CFU/ml)</th>
<th>Observation post challenge</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>3</td>
<td>4</td>
<td>5×10⁷</td>
<td>All survived</td>
<td>100% protection</td>
</tr>
<tr>
<td>G2</td>
<td>3</td>
<td>2</td>
<td>5×10⁷</td>
<td>2/3 survival</td>
<td>66.7% protection</td>
</tr>
<tr>
<td>G4 (+ve control)</td>
<td>3</td>
<td>2</td>
<td>5×10⁷</td>
<td>All survived</td>
<td>100% protection</td>
</tr>
<tr>
<td>negative control</td>
<td>3</td>
<td>0</td>
<td>5×10⁷</td>
<td>All died</td>
<td>No protection</td>
</tr>
</tbody>
</table>

Only calves vaccinated with higher dose per group were challenged this time. This is because the performance of lower vaccine doses was found poor during challenge for the blackleg vaccine component challenge trial. So animals which were vaccinated with lower volume per group were removed from the test animals.

Table 2. Challenge test result of Guinea pigs vaccinated with different vaccine doses.

<table>
<thead>
<tr>
<th>Features</th>
<th>G1 (4 ml)</th>
<th>G2 (2 ml)</th>
<th>G3 (positive control) (1 ml)</th>
<th>Control (1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death way</td>
<td>0</td>
<td>3 (30%)</td>
<td>1 (10%)</td>
<td>0</td>
</tr>
<tr>
<td>Serious subcutaneous/abdominal edema leading to wounding</td>
<td>0</td>
<td>2 (20%)</td>
<td>0</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>If animals with s/c or abdominal edema were euthanized as per recommendation, mortality rate was 50%</td>
<td>0</td>
<td>50%</td>
<td>10%</td>
<td>30%</td>
</tr>
<tr>
<td>Protection</td>
<td>100</td>
<td>50</td>
<td>90</td>
<td>70</td>
</tr>
</tbody>
</table>

Challenge test and result interpretation was made according to the pharmacopeia (http://ipc.nic.in/super/users/writecomm1main.asp). The seriously sick animals were euthanized to minimize suffering and considered as dead.

broth and agar, sabouraud agar. These results indicated that the vaccines were free from any bacterial and fungal contaminant and indicated also the vaccine to be adequately inactivated.

Vaccines safety test

Observation of all the animals vaccinated with the combined and monovalent vaccines indicated that the combined vaccines were safe except minor swelling observed at the injection site which completely subsides within 2 to 3 weeks. Except this minor local reaction, the vaccinated animals showed no abnormal reaction to the vaccines.

Immunogenicity of the trial vaccines

**Immunogenicity of hemorrhagic septicemia component of the combined vaccine**

**Challenge test:** The result of challenge test for the hemorrhagic septicemia component between 5th and 6th month post vaccination showed that the G1 combined vaccine given in 4 ml had 100% protection where out of three calves all (100%) were protected from the active challenge similar to the monovalent hemorrhagic septicemia vaccine while the G2 combined vaccine given in 2 ml per dose showed ⅔ (66.67%) protection only. Similar challenge made on the ninth month post-vaccination showed a 50% decline in immunogenicity for both combined and monovalent Hemorrhagic septicemia vaccine (Table 1). Calves vaccinated with G1 combined vaccine in 2 ml, G2 combined vaccine in 1ml and monovalent hemorrhagic septicemia vaccine in 1 ml were not challenged because these vaccine doses performed poorly in Blackleg component challenge test on guinea pigs.

Immunogenicity of the Blackleg component of the combined vaccine

The result of the immunogenicity test of the blackleg component of the combined vaccine showed that G1 combined vaccine had 100% protection in 4 ml per dose while the G2 combined vaccine had 90% protection in 2 ml amount. The monovalent Blackleg vaccine had also 100% protection to virulent *C. chauvoei* challenge. The other vaccination groups revealed a far less protection capability (Table 2).

Challenge tests for trial vaccines

The result for Blackleg component (Table 2) made us to
reject other lower vaccine doses and made us to give attention to G1 (4 ml), G2 (2 ml), G3 (2 ml) and negative controls only. As indicated for calf challenge in Table 1 for hemorrhagic septicemia component and guinea pig challenge in Table 2 for blackleg component, the challenge test result showed that G1 (4 ml) and G2 (2 ml) combined vaccines were better candidate combined vaccines. However, G1 (4 ml) had better performance than G2 (2 ml). Table 3 summarizes the result of both challenge tests and depicted that G1 in 4 ml combined vaccine to be the better performing trial combined vaccine group.

**Bacterial isolations**

Samples taken from jugular vein (whole blood), tissues (heart, liver, spleen, kidney and lymph nodes) and thoracic fluid from seriously sick or dead calves and cultures using routine bacteriological techniques and revealed re-isolation of the challenge organism, *P. multocida* type B:2. Similarly samples taken from muscel and edematouse abdominal fluid of severely ill guinea pigs and cultured anaerobically and tested by use of PCR revealed the reisolation of *C. chovoei*, as the challenge organism for blackleg component of the combined vaccine. This showed the re-isolation of organisms from sick animals or recently dead in both challenge components.

**DISCUSSION**

Haemorrhagic septicemia is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (OIE, 2012). Blackleg is an acute, febrile, highly fatal disease of cattle and sheep caused by *C. chauvoei* and characterized by emphysematous swelling, commonly affecting heavy muscles (*Clostridial myositis*) (Stampfli, 2016). These diseases have been known to be effectively prevented by vaccination with respective vaccines (Mosier, 2016; Stampfli, 2016). However, vaccination of animals by combining these two vaccines enables prevention of both diseases with a vaccine delivered via a single shot that reduces stress on the animals, financial, time and energy costs of the animal owner, and reduces chance of disease spread that could have been contracted via multiple times gathering of animals to vaccinate for HS vaccine and to vaccinate for blackleg vaccine at different times (Stämpfli, 2016; Johns and Hutter, 2010; Dodd, 2003; Thrusfield, 1995; Edwards, 1994).

In this trial, the safety and immunogenicity of combined hemorrhagic septicemia and blackleg vaccines formulated in two forms were evaluated in comparison to the respective monovalent hemorrhagic septicemia and blackleg vaccines. The result showed that the G1 combined vaccine given in 4 ml per dose gave 100% protection in hemorrhagic septicemia and blackleg component, the G2 combined vaccine in 2 ml per dose gave 66.7% protection in hemorrhagic septicemia component while 90% protection in blackleg component. The positive controls gave 100% protection for both hemorrhagic septicemia and blackleg vaccines. Ghanem and Ghanem (1987) reported the immunity conferred by combined vaccine of blackleg and hemorrhagic septicemia to be similar to that obtained by each vaccine alone. Other similar study made by Ardehali et al. (1997) proved the efficacy and safety of combined blackleg and hemorrhagic septicemia vaccines in both cattle and buffaloes. Srinivasan et al. (2012) in their study focused on evaluation of serological response of combined hemorrhagic septicemia, blackleg, foot and mouth disease, and rabies vaccines, found similar serologic response generated by combined vaccine and their respective individual components.

The challenge study in hemorrhagic septicemia component further indicated that the protection provided

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**Table 3. Challenge test result summary for trial vaccine groups.**

<table>
<thead>
<tr>
<th>Test</th>
<th>Vaccination group</th>
<th>Mono-valent blackleg vaccine</th>
<th>Monovalent vaccine</th>
<th>HS batch</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1 (4 ml) combined</td>
<td>G2 (2 ml) combined</td>
<td>batch BL32/12</td>
<td>PB17/12</td>
<td></td>
</tr>
<tr>
<td>Protection for blackleg component</td>
<td>100%</td>
<td>90%</td>
<td>100%</td>
<td>N/A</td>
<td>100% death</td>
</tr>
<tr>
<td>Protection for hemorrhagic septicemia component</td>
<td>100% b/n 5th and 6th month vaccination</td>
<td>66.7% b/n 5th and 6th month vaccination</td>
<td>N/A</td>
<td>100% b/n 5th and 6th month vaccination</td>
<td>100% death</td>
</tr>
<tr>
<td></td>
<td>50% b/n 9th and 10th months vaccination</td>
<td>50% b/n 9th and 10th months vaccination</td>
<td>N/A</td>
<td>50% b/n 9th and 10th months vaccination</td>
<td>100% death</td>
</tr>
</tbody>
</table>

N/A is not applicable
is 100% between 5th and six month challenge while these figure falls to 50% at challenge made between 9 and ten months post vaccination. Similarly, Hanna et al. (2014) reported higher log protection against combined clostridia and pasteurella vaccines at six months than 7 months in sheep.

When the result of this study is seen over-whole, the group one vaccine in 4 ml/dose showed the better performance than group 2 combined vaccines in 2 ml/dose, even though the protection showed by group 2; 2 ml combined vaccine is also acceptable according to Indian pharmacopoeia monograph (http://ipc.nic.in/super/users/writecomm1main.asp?id=508 &cuid=&Hid). A literature source on different manufacturers reveals that the blackleg and hemorrhagic septicemia combined vaccine is given in 4 ml amount per dose in cattle and buffaloes by Indian immunological limited (Raksha Vaccine). In their study on the relationship between the response of guinea pigs and sheep following vaccination and challenge with virulent C. chauvoei, Crichton et al. (1986) found guinea pig laboratory model to be a valid indicator of field performance for vaccines containing blackleg antigen.

Therefore, according to the data generated by these experiments, the institute can produce combined vaccine for blackleg and hemorrhagic septicemia vaccine which could be given in 4 ml/dose in cattle after performing field level safety and immunogenicity tests.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**REFERENCES**


Indian pharmacopoeia commission (http://ipc.nic.in/super/users/writecomm1main.asp?id=508 &cuid=&Hid).


