CHARACTERIZATION AND GROWTH EVALUATION OF *STREPTOCOCCUS EQUI* IN HORSES: A PREDICTORY APPROACH TOWARDS ITS MANAGEMENT IN HERDS

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ABSTRACT: Strangles is the most common infectious and contagious disease of equines that affects upper respiratory tract. It is prevalent among the horses in sub-continent from pre partitioned times, an effective study to isolate locally prevalent strains in the area and study of factors affecting their consequent growth is essential. For the said purpose, twenty nasal swab samples were collected, ten from clinically sick and ten from apparently healthy horses with the history of disease. Isolated strains were undergone identification through biochemical and lancefield testing. Maximum in-vitro growth of *Streptococcus equi* (S. equi) is a critical factor for its cost effective bioceutics production. Effect of different factors like composition of medium such as (Tryptic Soy Broth (TSB), Nutrient Broth, Brain Heart Infusion broth (BHI), Reinforced Clostridial Medium (RCM) and Peptone Broth, pH (5, 7, 9), gas (oxygen availability, 5% CO2, aeration), horse serum (2%, 5%, 10%) and temperature (35°C, 37°C, 39°C) on growth of *S. equi* was therefore carried out. Growth was expressed in form of Colony Forming Units (CFU) which was converted into log values followed by statistical analysis. TSB among media, pH 7, CO_2 5% gas, horse serum 5% and incubation temperature 37°C showed optimum growth of *S. equi*.

Key Words: *Streptococcus equi*, API testing, lancefield classification, colony forming unit, optimum growth, media, serum, gas, temperature.

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INTRODUCTION

Equines are major part of livestock sector in Pakistan and are estimated to be consisting of 5.7 Million Asses, 0.4 Million horses and 0.2 Million mules (Pakistan Economic Survey 2021-2022). Strangles is one of the most important diseases and is responsible for high economic loss in equine industry. More than 30% of equine infectious disease episodes are because of strangles. Disease is found in horses of all ages, but is more common in foals where prevalence reaches up to 87% (Ijaz *et al*. 2012).

Animals suffering from strangles exhibit signs of depression, anorexia, fever (104°C), soft cough and serous nasal discharge which progressively become mucopurulent. The swollen submandibular and retropharyngeal lymph nodes cause pain and obstruction in breathing (Sweeney *et al*. 2005). Lymph nodes usually burst out in severe conditions (Gutiérrez 2013).

An effective vaccine can provide immense protection against this debilitating disease. Previously developed recombinant vaccines which contain only few antigens, are not only expensive but also cause undesirable reactions at site of administration and confer temporary immunity (Nascimento and Leite 2012). A vaccine containing maximum possible antigens is more efficient in triggering immune response to a protective level, thus presence of all possible immunogens or enhancement in the level of immunogen per dose of vaccine should be ensured (Julik and Reyes-del Valle 2016), (Lee *et al*. 2016). Physical factors like temperature, aeration, duration of growth and physical light etc. and chemical factors like nutritional components of growth medium, pH, gaseous requirement modulate in-vitro growth of bacteria (Sarwar *et al*. 2013). Sufficient biomass/CFU is essential for the production of quality biologics and diagnostics.

This paper encompasses the research in which appropriate diagnosis of strains prevalent in the Pakistan is developed and factors affecting biomass production of these strains considered. Such study will assist in production of the vaccine for protecting the local population of horses against highly detrimental disease, in future.

MATERIALS AND METHODS

Animal Selection: Samples from twenty horses subjected to this study were collected from a local stud farm in Sargodha. Ten horses with purulent nasal discharge or lymph node swelling were selected similarly ten horses with the history of clinical signs were included in the study. Sampling was done on complete randomized method (Ijaz *et al*. 2012). ATCC culture Streptococcus equi was used for comparison in the study.

Sample collection and transportation: Samples were collected from nasal cavities (Lindahl *et al*. 2013) with the help of cotton swabs containing Amies transport media (Teese *et al*. 2003).

Culture and biochemical tests: All the samples were swabbed over blood agar containing 5% sheep blood agar v/v and incubated anaerobically at 37°C for 24 hours (Petts 1984). Presence of round mucoid beta-hemolytic colonies is characteristic of *S. equi* (Gera and McIver 2013*),* these were proceeded further for colony morphology, gram staining and catalase testing. Complete biochemical profiles of bacteria were evaluated by using Analytical Profile Index (API) kit (Biomerieux) by following the manufacturer's instructions.

Lancefield grouping: Lancefield classification test to classify the bacterial samples into different groups was performed with help of PathoDxtra Strep Grouping kit according to manual's instruction. Appearance of agglutination was termed as positive whereas no agglutination was considered negative.

Factors affecting growth of *S. equi:* Effect of different parameters, such as different media, pH, gas availability, horse serum concentration and varying values of temperature on growth of *S. equi* were studied (Mahmood 2001; Shah *et al*. 2008).

Effect of Medium: Effect of five media such as Tryptic Soy broth (TSB), Nutrient Broth, Brain Heart Infusion broth (BHI), Reinforced Clostridial Medium (RCM) and Peptone Broth was evaluated. Each of the medium were prepared according to the manufacturer's instructions (Sarwar *et al*. 2013) and proceeded in triplicate.

Effect of pH: Effect of 5, 7 and 9 pH of TSB medium (in triplicate) was observed on the growth of *S. equi*. 0.5N HCl and 0.5N NaOH were used to decrease and increase the pH of medium respectively (Yang *et al*. 2018).

Effect of Gas: Gas effect was studied by incubating with 5% CO_2,by incubating in a shaking incubator at 500 rotations per minute and by incubating in an aerobic incubator. The temperature of all incubators was kept constant at 37°C (Shimamoto *et al*. 1990).

Effect of Horse serum: Serum effect was studied by using commercially available horse serum, media was augmented and tested with 2%, 5% and 10% serum concentration (Erickson and Norcross 1975).

Effect of Temperature: Growth pattern of *S. equi* was studied at 35°C, 37°C and 39°C for 24 hours (Shah *et al*. 2008).

Total Viable Count: To study the effect of above factors on growth of *S. equi*, Pour plate method was performed by preparing appropriate dilutions in autoclaved normal saline and then adding defined quantity of dilution to molten blood agar. After solidification, the blood agar plates were incubated at 37°C for 24 hours (Sanders 2012).

Statistical Analysis: CFU data in exponential form of each experiment was transformed into continuous data (log values) and then analyzed statistically by One-way Analysis of Variance using SPSS v.20 (Chaudhry 2011).

RESULTS

Out of twenty horses, 5 samples were found positive for *Streptococcus equi*, two were from clinically infected horses and three were recovered from apparently healthy animals making the recovery rate 25%.

Culture and biochemical tests: Samples were swabbed over blood agar different types of colonies were observed with different hemolytic pattern. Cultures exhibited typical biochemical profile as shown in table 1.

Table 1. Depiction of results obtained from various clinical tests by *S. equi*

Test	Color Observed	Interpretation
VP	Colorless	Negative
HIP	Colorless	Negative
ESC	Colorless	Negative
PYRA	Colorless	Negative
aGAL	Colorless	Negative
bGUR	Blue	Positive
bGAL	Colorless	Negative
PAL	Violet	Positive
LAP	Orange	Positive
ADH	Red	Positive
RIB	Red	Negative
ARA	Red	Negative
MAN	Red	Negative
SOR	Red	Negative
LAC	Red	Negative
TRE	Red	Negative
INU	Red	Negative
RAF	Red	Negative
AMD	Yellow	Positive
GLYG	Yellow	Positive
VP	Colorless	Negative

Lancefield grouping: All the beta-hemolytic colonies gave agglutination (positive reaction) with Group C specific antisera when tested for Lancefield classification using Patho dxtra Strept Grouping kit (Thermo Scientific) as shown in Figure 1.

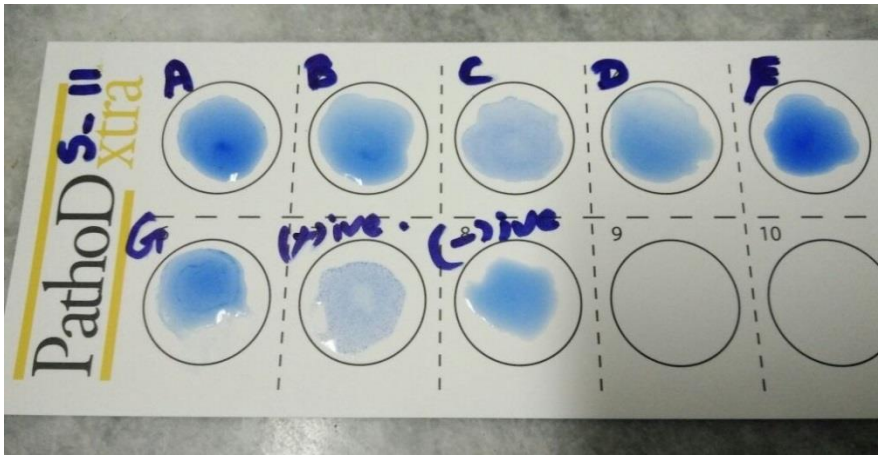

Figure 1. Results of lancefield grouping (A, B, D, F, and G showed no clumping, C showed clumping, (-)ive is negative control and (+)ive is positive control)

Effect of media composition: Maximum bacterial growth was observed in both case of BHI broth and RCM being $5x10^9$CFU/ml and $4x10^9$ CFU/ml while least being Peptone broth with only 3 CFU/ml. TSB and Nutrient Broth gave $8x10^7$ CFU/ml and $3x10^4$ CFU/ml respectively as shown in Figure 2.

Effect of pH: Maximum biomass was obtained from TSB with pH 7 being $3.8x10^7$CFU/ml, whereas it was $5.8x10^6$CFU/ml for pH 5 and $6.9x10^6$ CFU/ml for pH 9 as shown in Figure 3.

Effect of gas (oxygen availability): The biomass of *S. equi* for CO_2, aerobic (still culture) and shaking culture environments were found to be as $2.6x10^9$ CFU/ml, $3.9x10^7$ CFU/ml and $3.4x10^7$ CFU/ml respectively depicted in Figure 4.

Effect of horse serum: $1.6x10^9$ CFU/ml, $3.4x10^{11}$ CFU/ml and $3.9x10^{11}$ CFU/ml concentrations were obtained for 2%, 5% and 10% horse serum respectively as shown in Figure 5.

Effect of Temperature: Growth of *S. equi* in TSB at 35°C, 37°C and 39°C turned out to be $1.5x10^7$CFU/ml, $7.9x10^7$ CFU/ml and $7.4x10^6$CFU/ml respectively (Figure 6). *S. equi* grows favorably between 35°C till 37°C but further increase in temperature does not efficiently support growth of bacterium in medium.

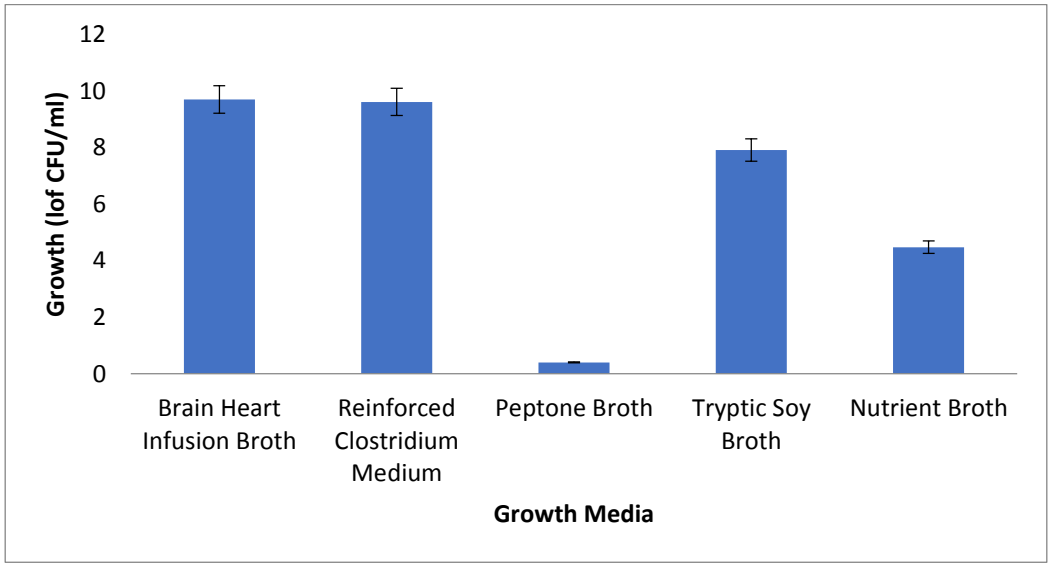

Figure 2. Effect of media on *in-vitro* growth of *S. equi*

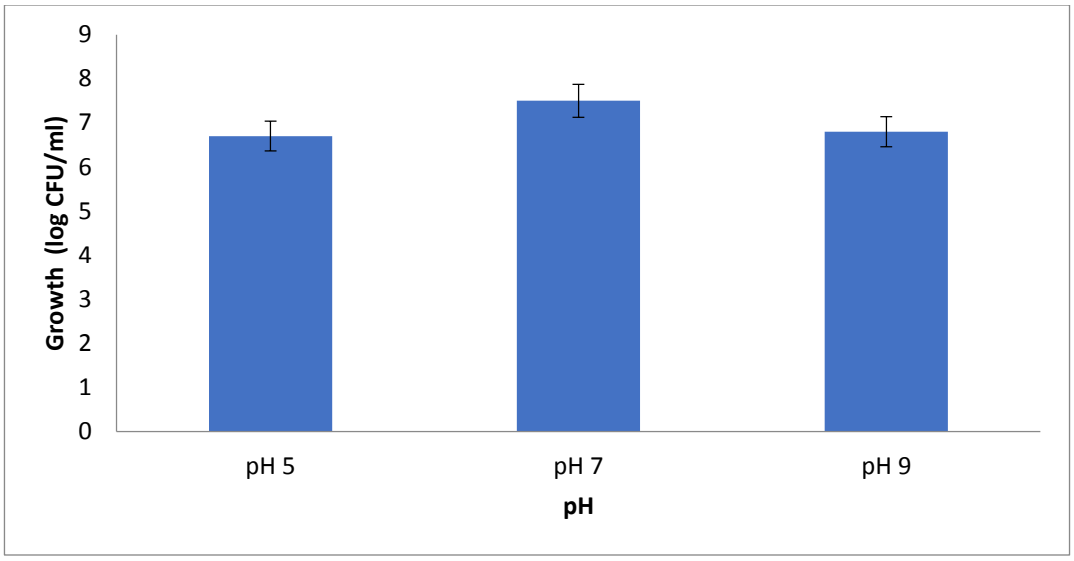

Figure 3. Effect of pH on *in-vitro* growth of *S. equi*

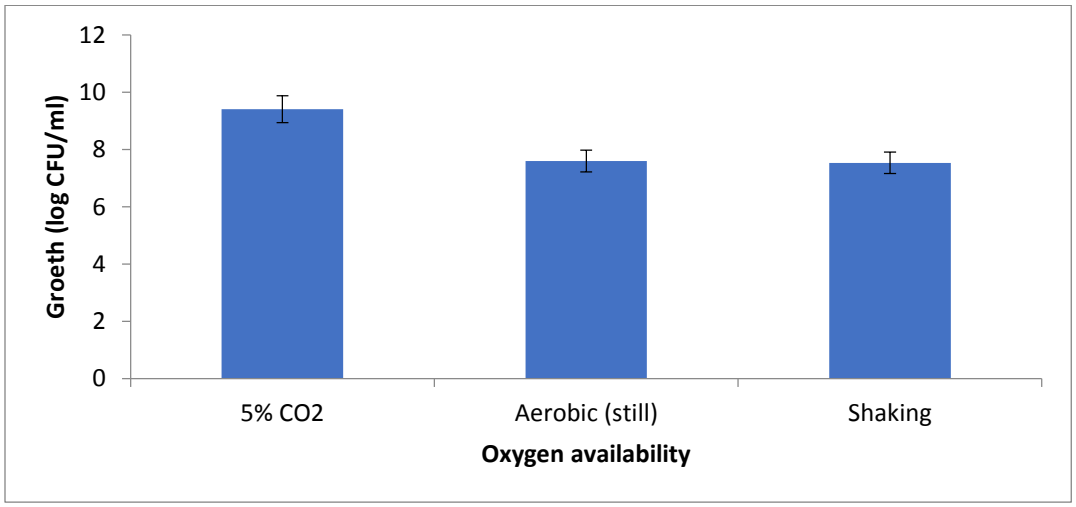

Figure 4. Effect of gas on in-vitro growth of S. equi

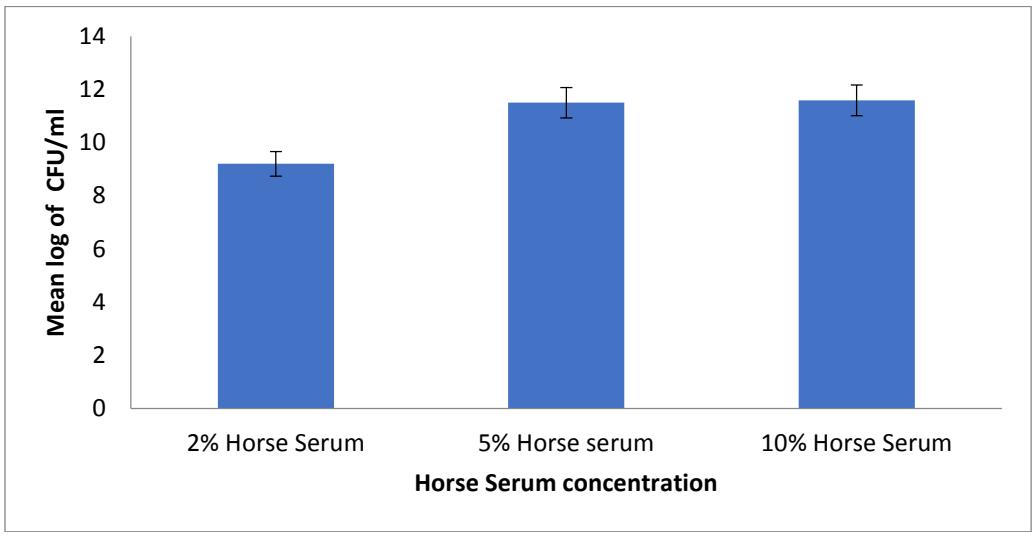

Figure 5. Effect of horse serum on *in-vitro* growth of *S. equi*

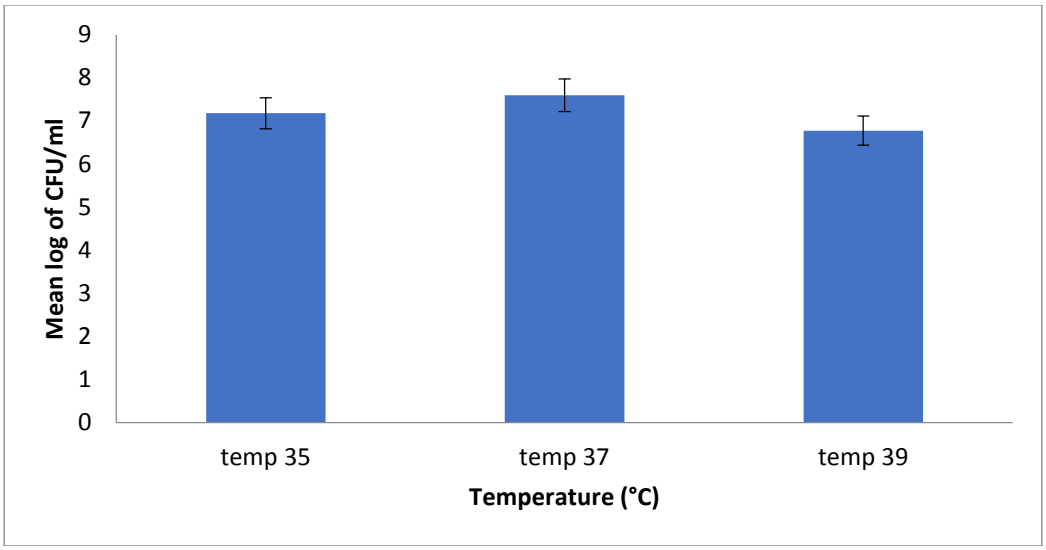

Figure 6. Effect of temperature on *in-vitro* growth of *S. equi*

Cluster analysis of bacterial activity against *in-vitro* growth conditions: As shown in Figure 7, bacterial activity was observed for temperature, pH, horse serum concentration, available gas and growth media. Maximum growth of S. equi was observed in case of temperature (37°C), gas availability (5% CO_2) and pH (7.0), while minimum growth was observed in case of pH (5.0) and temperature (39°C). Other parameters showed an appropriate response against bacterial growth.

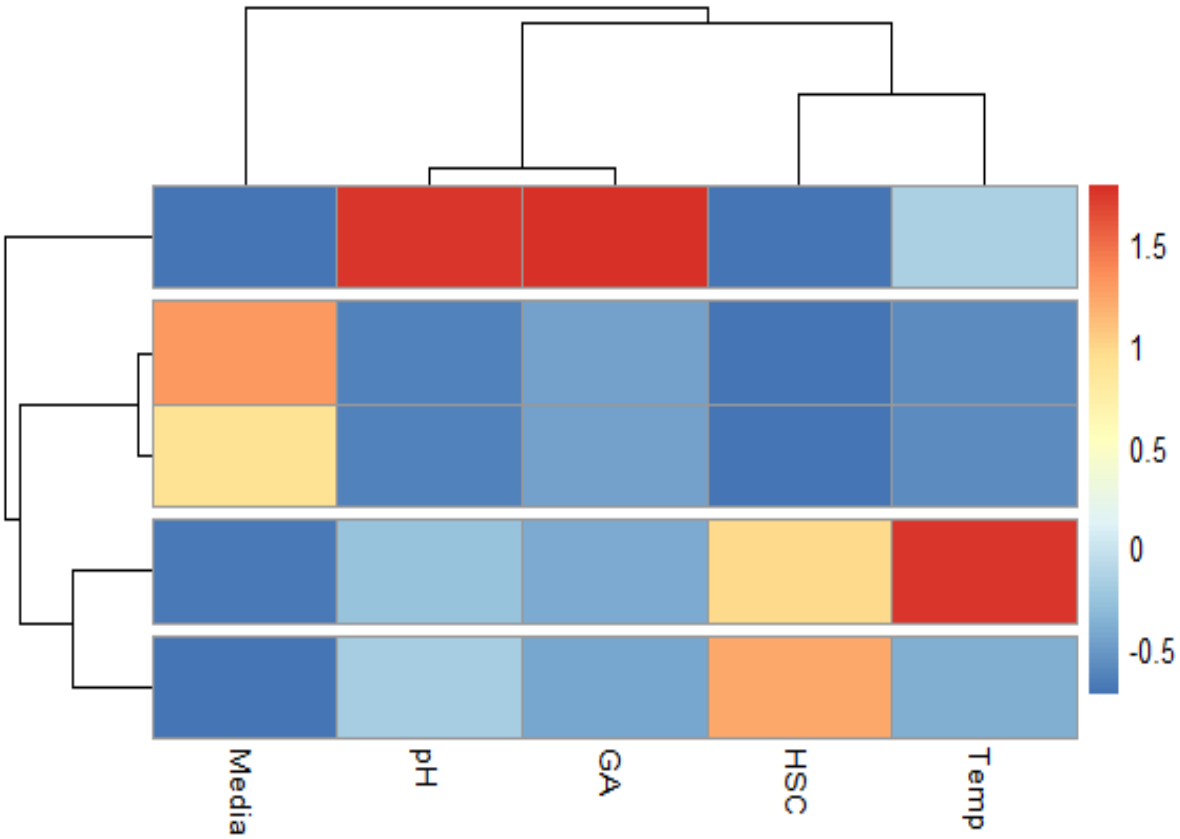

Figure 7. Cluster analysis of tested parameters against *S. equi*. The cluster analysis of all parameters determining the bacterial growth in heat map form. The color scale depicts the extent of growth corresponding single parameter.

Relationship among the tested *in-vitro* parameters: The correlation analysis results identified a significant correlation with bacterial activity. As shown in Figure 8, it was found that pH showed a positive correlation with media in response to bacterial growth. Gas availability has a positive correlation with media as bacterial growth was at exponential rate. Horse serum content was negatively correlated with media and temperature showed positive correlation with media. pH is positively correlated with gas availability but a negative correlation was observed in case of horse serum concentration and temperature, respectively. Horse serum concentration and gas availability showed negative correlation. Temperature and gas availability were negatively correlated while horse serum content and temperature showed positive correlation. Thus, correlation proves that all parameters have significant performance in response to bacterial growth at a 5% level of significance ($p \leq 0.05$).

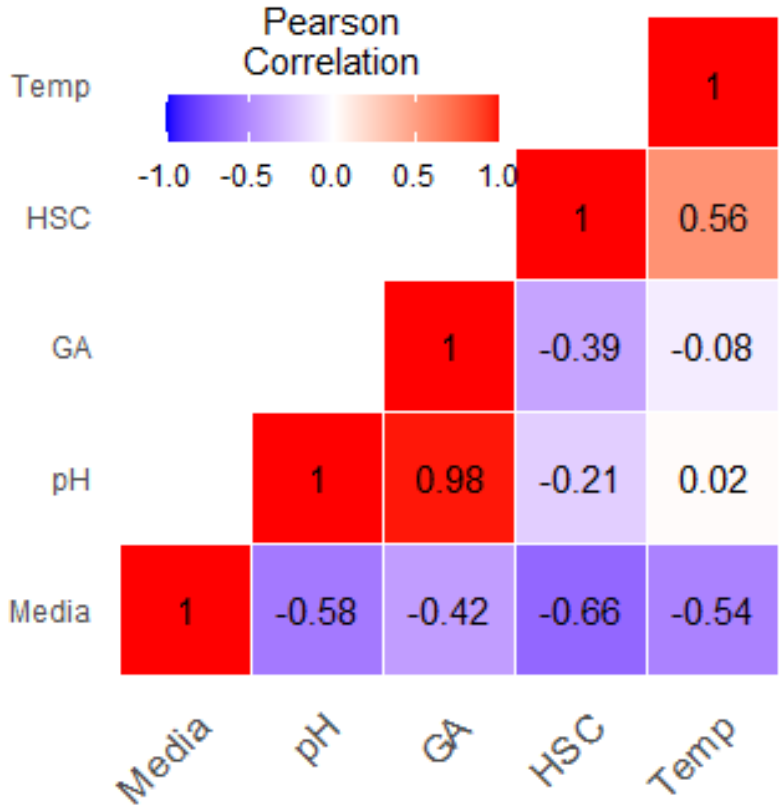

Figure 8. Correlation analysis among the tested parameters against *S. equi*. The digits represent r-value (correlation value) according to the scale of the Pearson's correlation mentioned above the matrix.

DISCUSSION

Equines play an important role in economy of a country but disease outbreaks cause heavy losses. Disease like Strangles with high morbidity is difficult to control. Strict measures can only be taken in an effective way when proper diagnosis of the disease is made. This study therefore was designed to encompass the isolation of bacterium from field conditions and its laboratory based diagnosis.

Through sample collection from nasal mucosa of horses, its conventional culturing and biochemical tests, streptococcus was recovered from 25% of the samples. Strangles has been reported in horses of different districts of Punjab from pre-partition time (Minett at al, 1944) with the prevalence rate of 45.2%. Young horses with age less than 2 years are more prone to this disease as prevalence rate reaches to 87% in this group. For the recovery of etiologic agent of Strangles, horses with respiratory distress are used for the sample collection, horses affected from Strangles show signs that typically involve biphasic fever, purulent nasal discharge, fever, cough and submandibular lymph node swelling. Sample from nasal cavity either through Nasal swabs or Nasal washes are also effective for its isolation (Ijaz *et al*. 2012). Bacteria cannot be recovered from nasal cavity until 24-48 hours post pyrexia. Nasal swabs is commonly used method however nasal washes are better source of bacterial recovery because it provides greater contact area. Maximum bacteria can be recovered from lavages

collected from Guttural pouch with the help of endoscope as bacteria normally persist in guttural pouch even after disappearance of clinical signs (Sweeney *et al*. 2005; Ghamdi 2008; Newton *et al*. 2000).

Streptococcus equi is very fastidious and slow growing bacteria, further growth of other bacteria in respiratory tract limitise its growth. *Streptococci* are gram positive bacteria that appear spherical in shape. *Streptocuucs equi* is facultative anaerobe it doesn't need oxygen for its growth however it has enzyme that helps it to tolerate the Oxygen. Streptococcus has manganese dependent superoxide dismutase that converts reactive oxidative species to H_2O_2 (Weisiger and Fridovich 1973; Hardie and while 1995). It lacks Catalase activity but has peroxidase that reduces H_2O_2 to H_2O by using NADH as electron acceptor (Gibson *et al*. 2000). This bacteria is unable to ferment Trehalose, Sorbitol, Lactose and Ribose (Keely *et al*. 2006; Kuwamoto 2001).

An effective vaccine administration is necessary for upholding protective immunity levels against Strangles. The lack of required level of immunogens, toxins and bacterial biomass are incriminated for the failure of immunprophylaxis. A vaccine containing maximum possible antigens will be more efficient in triggering immune response to a protective level (Nascimento and Leite 2012). Due to this reason, the study of growth patterns and requirements was taken into account.

Chemical components of BHI, RCM and TSB supported maximum growth of *S. equi* among media; but BHI and RCM are not appropriate to be used for vaccine production as they contain animal origin components which may cause an allergic response to the host post-vaccination (Carolina Biological Supply Company, 2012). These are, however, routinely employed to obtain high density biomass for production of biological diagnostics (Bhatti 2005;Hussain 2013). TSB has no such component which may cause harm to the host, therefore, TSB is deemed suitable for vaccine production studies. It can also be enriched with blood, serum or any other nutrient required by fastidious microbes for their growth without affecting the capability of medium components. pH 7 supported optimum growth of *S. equi.* These findings are in accordance with fact that streptococci survive neither in acidic nor in basic conditions. Streptococci only survive for longer time periods with pH range close to neutral (6.5-7.5) (Savic and McShan 2012). *S. equi* do not produce certain enzymes which are essential for respiratory energy production biochemical pathways, therefore the favorable mode of energy production is through fermentation of carbohydrates. Due to this reason *S. equi* prefers growth in microaerophilic to anaerobic conditions. Although its facultative anaerobic nature allows it to grow in aerobic conditions but slowly. Total biomass of *S. equi* grown in 5% CO_2 is higher as compared to aerobic condition, thus presence of 5% CO_2 condition supports optimal growth of *S. equi* and growth of *S. equi* in flasks settles down where oxygen content is minimal (Gera and McIver 2013). For a vaccine to be effective, the main immunogens responsible for causing disease must be included. Likewise, S. equi utilizes both extracellular and cellular weapons to enter host animal and cause disease. It is important that all extracellular and cellular components be expressed during growth. Enrichment of broth with horse serum ensures that all inducible extracellular enzymes also be produced which will play utmost important role in conferring immunity if included in vaccines. Optimum biomass was observed when medium was enriched with 5% heat inactivated horse serum (Hulting *et al*. 2009). Growth of *S. equi* is optimum at 37°C but slows down at 35°C. At higher temperature, growth decreases due to increased evaporation of moisture from medium prevents further multiplication of bacterial cells (Weese *et al*. 2009).

Conclusion: It was concluded that TSB, pH 7, CO_2 5%, horse serum 5% and 37°C temperature is critical for optimum production of biomass and its toxins in in-vitro culture. This knowledge will greatly aid in obtaining optimum growth to increase total dose production volume while utilizing minimum resources and therefore, the production of a cost-effective vaccine be made possible.

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