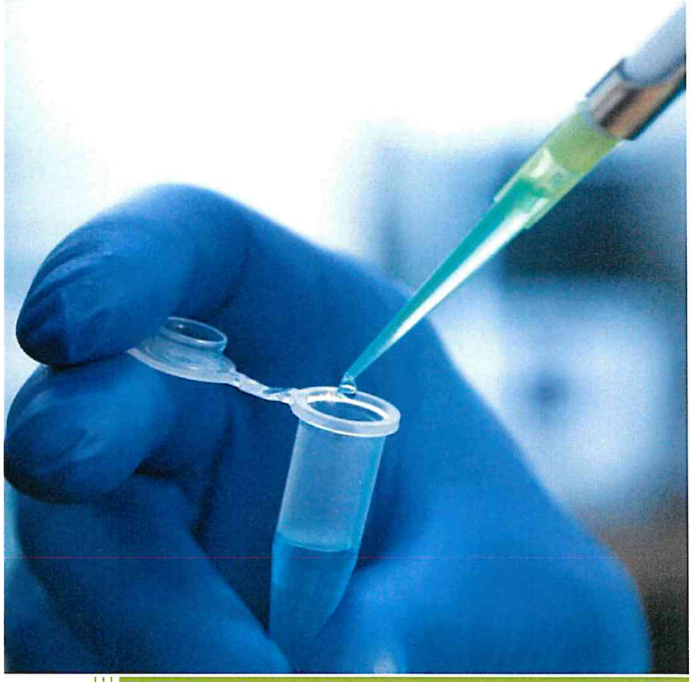
2020

Equine BIO Fluid –Efficacy Trial

Phase 2, Step 1 Testing

Carried out on Behalf of Equine Bio Genie LTD



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PROTECTING ANIMAL HEALTH

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Abbreviations

ATCC	American Type Culture Collection
EBF	Equine BIO Fluid
NCTC	National Collection of Type Cultures
SOP	Standard operating procedure
TNTC	Too numerous to count
NCPF	National Collection of Pathogenic Fungi

1. Scope

1.1. The aim of this protocol was to assess the efficacy of Equine BIO Fluid (EBF) based on EN 1656:2019 and EN 1657:2016, using equine field strains. The isolates were sourced from equine clinical samples received by the Irish Equine Centre during 2020. The following strains were used for the purpose of this trial:

1.1.1. *Streptococcus equi* (Laboratory no: 20MI010878)

1.1.2. *Aspergillus niger* (Laboratory no: 20EN004535)

1.1.3. *Trichophyton sp.* (Laboratory no: 20MI0025171)

2. Selection of Method

2.1 Testing of Equine BIO Fluid (EBF) was carried out based on the following standards:

- European Standard EN 1656:2019 – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in the veterinary area – Test method and requirements (Phase 2, Step 1).
- European Standard EN 1657:2016 – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in the veterinary area – Test method and requirements (Phase 2, Step 1).

2.2 The European Standards, EN 1656:2019 and EN 1657:2016, describe suspension tests for establishing whether a chemical disinfectant has or does not have bactericidal / fungicidal / yeasticidal activity under laboratory conditions which influence the action of the disinfectant in practical use, e.g. organic material. The type and level of the interfering substance, in this case, equine serum (5% on reaction mix – considered moderate level soiling) were agreed with the client before testing commenced. The laboratory conditions under which testing was carried out, e.g. concentrations of disinfectant product tested, testing temperature, contact times etc. were established with the client before testing commenced. The method carried out involved neutralisation of the bactericidal / yeasticidal activity by dilution into a neutraliser (see Section 10).

2.3 The European standard, EN 1656:2019 states, "applies to products that are used for equipment disinfection by immersion, surface disinfection by wiping, spraying, flooding or other means and teat disinfection in the veterinary area – e.g. in the breeding, husbandry, production, veterinary care facilities, transport and disposal of all animals except when in the food chain following death and entry into processing industry". EN 1657:2016 states, "applies to products that are used in the veterinary area e.g. in the breeding, husbandry, transport and disposal of all animals except when in the food chain following death and entry to the processing industry". For this reason, the standards EN 1656:2019 and EN 1657:2016 were deemed appropriate for use in this trial.

3. Test Organisms

3.1 All strains were sourced from equine clinical samples submitted to The Irish Equine Centre in 2020 and have been stored in a secure bank for further analysis if deemed necessary. The following equine field strains were used for the purpose of efficacy testing in this trial:

3.1.1 *Streptococcus equi* (Laboratory reference number – 20MI010878)
3.1.2 *Aspergillus niger* (Laboratory reference number – 20EN004535)
3.1.3 *Trichophyton sp* (Laboratory reference number – 20MI0025171)

4. Concentrations

4.1 The testing concentrations of EBF were previously agreed with the client before testing commenced. They were chosen to be 1:5, 1:16 and 1:60 ratio of EBF to sterile hard water (See Section 9, Point 9.2).

5. Contact Times

5.1. Contact periods of EBF with the test surface were at the following times:

- 1 minute ± 5 s
- 5 minutes ± 10 s
- 15 minutes ± 10 s
- 1 hour ± 10 s
- 6 hours ± 10 s
- 24 hours ± 10 s

6. Test Temperature

6.1. Testing was carried out at room temperature 22°C ± 1°C.
6.2. The temperature was monitored daily using a calibrated thermometer and recorded onto the Microbiology laboratory temperature logbook, (Worksheet W1.002 - Temperature Log).

7. Interfering Substance

7.1 The interfering substance was chosen to be equine serum (5% on reaction mix).
7.2 The equine serum was sourced from clinical equine samples received by the Irish Equine centre.
7.3 The horse serum was sterilised by filtering with a 0.45µm filter before use.

8. Test Conditions

8.1 Tests were carried out under clean and dirty conditions.
8.2 Clean conditions constituted a mix of the bacterial or fungal suspension mix and EBF.
8.3 Dirty conditions constituted a mix of the bacterial or fungal suspension mix, horse serum (5% on reaction mix) and EBF.

9. Hard Water

9.1 Hard water (See Section 9, Point 9.2) was used at all stages of dilution throughout the entire phase of testing.

9.2 The hard water was made according to EN 1656 as follows:

- Solution A – 19.84 g $MgCl_2$ and 46.24 g $CaCl_2$ were weighed out and made up to 1000 ml with water. This solution was sterilised by autoclaving at 121°C for 15 minutes at 15 psi.
- Solution B – 35.02 g $NaHCO_3$ was made up to 1000 ml with water and sterilised by membrane filtration (using 0.45 µm pore size filter).
- 6 ml of Solution A was added to 600 ml of water in a sterile 1000 ml container. 8 ml of Solution B was then added to the sterile container and made up to 1000 ml with water.
- The final pH of the solution was 7.0 ± 0.2.
- The sterile hard water was prepared on the day of use.

10. Inactivator

10.1 The inactivation liquid used was prepared as follows:

- Lecithin – soya: 3 g
- Tween 80: 30 ml
- Sodium thiosulphate: 5 g
- L-histadine: 1 g
- Phosphate buffer: 10 ml

10.2 The above reagents were weighed out in a 1000 ml container and made up to 1000 ml with water and sterilised at 121°C for 15 minutes at 15 psi.

11. Media Used

12.1 Columbia blood agar (Oxoid) was used to test EBF against *Streptococcus equi*.

12.2 Sabouraud Dextrose agar (Oxoid) was used to test EBF against *Aspergillus niger* & *Trichophyton sp*.

12.3 All media was prepared in house according to SOP P5.006 (P5.006 (Revision 05) - Preparation of routine culture media). Records are maintained and stored in the Microbiology Unit, Room no. 89, (Worksheet W5.004 – (Revision 04), Microbiology media prep. record sheet).

12. Test Procedure

12.1 Working Culture of the Bacterial Test Organisms

12.1.1 A subculture of the stock culture was prepared by plating out on Columbia blood agar.

12.1.2 The Columbia Blood agar plates were incubated at 37°C ± 1°C for 24 hours.

12.1.3 After 24 hours, a second subculture was made from the Columbia Blood agar plates and incubated at 37°C ± 1°C for 24 hours. This was the working culture of the test organism.

12.2 Working Culture of the Fungal Test Organism

12.2.1 The working culture was prepared by sub-culturing from the stock culture and plating on Sabouraud Dextrose agar.

12.2.2 The Sabouraud Dextrose agar plates were incubated at 22°C ± 1°C for 72 hours for *Aspergillus niger* and in the case of the *Trichophyton sp* for 7 days.

12.2.3 After 72 hours, a second subculture was made from the Sabouraud Dextrose agar plates and incubated at 22°C ± 1°C for 72 hours for *Aspergillus niger* and in the case of the *Trichophyton sp* for 7 days. This was the working culture of the fungal test organism.

12.3 Bacterial Test Suspension

12.3.1 Using an inoculating loop, bacterial colonies were transferred from the working culture plates to a universal containing 20 ml of sterile hard water. This was the bacterial working stock solution.

12.3.2 Using a vortex, a homogenous mixture was obtained from which a Miles and Misra test could be performed to achieve the log value of the bacterial test suspension.

12.4 Fungal Test Suspension

12.4.1 Using an inoculating loop, fungal colonies were transferred from the working culture plates to a universal containing 20 ml of sterile hard water. This was the fungal working stock solution.

12.4.2 Using a vortex, a homogenous mixture was obtained from which a Miles and Misra test could be performed to achieve the log value of the fungal test suspension.

12.5 Product and Control Test Solutions

12.5.1 A clean mix was prepared from either the bacterial suspension (Point 12.3 above) or the fungal suspension (point 12.4 above)- 6mls of the bacterial or fungal suspension was added to 4mls sterile hard water.

12.5.2 A clean control was prepared by adding 2.5 ml of clean mix (Point 12.5.1) to 2.5 ml of sterile hard water.

12.5.3 A dirty mix was prepared by adding 2 ml of bacterial or fungal suspension to 3 ml of sterile hard water and 5 ml of filtered horse serum.

12.5.4 A dirty control was prepared by adding 1 ml of dirty mix (Point 13.5.3) to 9 ml of sterile hard water.

12.5.5 **Equine BIO Fluid (EBF) 1:5** under **clean** conditions: A 1:5 dilution of the neat product was carried out first (20mls of neat disinfectant was added to 80mls sterile water). 2.5 ml of the 1:5 dilution was transferred to a universal to which then, 2.5 ml of the clean mix (point 12.5.1) was added. (Once the clean mix was added the timer was started to observe the contact times, see Section 5).

12.5.6 **Equine BIO Fluid (EBF) 1:16** under **clean** conditions: A 1:16 dilution of the neat product was carried out first (5mls of neat disinfectant was added to 75mls sterile water). 2.5 ml of the 1:16 dilution was transferred to a universal to which then, 2.5 ml of the clean mix (point 12.5.1) was added. (Once the clean mix was added the timer was started to observe the contact times, see Section 5).

12.5.7 **Equine BIO Fluid (EBF) 1:60** under **clean** conditions: A 1:60 dilution of the neat product was carried out first (2mls of neat disinfectant was added to 118mls sterile water). 2.5 ml of the 1:60 dilution was transferred to a universal to which then, 2.5 ml of the clean mix (point 12.5.1) was added. (Once the clean mix was added the timer was started to observe the contact times, see Section 5)

12.5.8 **Equine BIO Fluid (EBF) 1:5 under dirty** conditions: 9 ml of the 1:5 dilution of the neat product was transferred to a universal to which then, 1 ml of the dirty mix (point

12.5.3) was added. (Once the dirty mix was added the timer was started to observe the contact times, see Section 5).

12.5.9 **Equine BIO Fluid (EBF) 1:16 under dirty** conditions: 9 ml of the 1:16 dilution of the neat product was transferred to a universal to which then, 1 ml of the dirty mix (point 12.5.3) was added. (Once the dirty mix was added the timer was started to observe the contact times, see Section 5).

12.5.10 **Equine BIO Fluid (EBF) 1:60 under dirty** conditions: 9 ml of the 1:60 dilution of the neat product was transferred to a universal to which then, 1 ml of the dirty mix (point 12.5.3) was added. (Once the dirty mix was added the timer was started to observe the contact times, see Section 5)

12.5.11 At the observed contact times of 1 minute, 5 minutes, 15 minutes, 1 hour, 6 hours and 24 hours, 1 ml of each of the solutions (points 12.5.5 to 12.5.10) was transferred to a universal containing 9 ml of inactivation fluid (See Section 10).

12.5.12 After a 15-minute period in inactivation fluid, 10 µl of the fluid (point 12.5.12) was plated onto Columbia Blood agar plates (for the bacterial test suspensions) and Sabouraud Dextrose agar plates (for the fungal test suspensions) in duplicate.

12.5.13 The Columbia blood agar plates were incubated at 37°C ± 1°C for 24 hours and then examined for the presence of bacterial growth.

12.5.14 Sabouraud Dextrose agar plates were incubated at 22°C ± 1°C for 72 hours for *Aspergillus niger* and in the case of the *Trichophyton sp* for 7 days and then examined for the presence of fungal growth.

12.5.15 Any plates showing growth were calculated to produce Log values for the organism indicated. Any plates with >150 colonies were deemed TNTC (Too Numerous To Count).

13. Pass Criteria

13.1 The product should demonstrate at least a 4 decimal log reduction in the target organism to be deemed a satisfactory result.

13.2 All control plates must show expected criteria.

14. Results

Table 14.1: Test results showing log counts of *Streptococcus equi* (Ref 20MI010878)
Date of trial: 21/07/2020

Equine BIO Fluid Concentration	Contact Times					
	1 min	5 mins	15 mins	1 hour	6 hours	24 hours
1:5 Clean Conditions	0cfu	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:16 Clean Conditions	TNTC	TNTC	0 cfu	0 cfu	0 cfu	0 cfu
1:60 Clean Conditions	TNTC	TNTC	TNTC	0 cfu	0 cfu	0 cfu
1:5 Dirty Conditions	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:16 Dirty Conditions	TNTC	TNTC	0cfu	0 cfu	0 cfu	0 cfu
1:60 Dirty Conditions	TNTC	TNTC	TNTC	0 cfu	0 cfu	0 cfu

Bacterial Test Suspension: Log 7.31. TNTC = Too numerous to count

Table 14.2: Test results showing log counts of *Aspergillus niger* (Ref 20EN004535)
Date of trial: 01/10/2020

Equine BIO Fluid Concentration	Contact Times					
	1 min	5 mins	15 mins	1 hour	6 hours	24 hours
1:5 Clean Conditions	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:16 Clean Conditions	Log 5.81	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:60 Clean Conditions	Log 6.16	Log 6.23	Log 5.70	0 cfu	0 cfu	0 cfu
1:5 Dirty Conditions	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:16 Dirty Conditions	Log 5.81	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:60 Dirty Conditions	Log 6.48	Log 6.13	Log 5.88	0 cfu	0 cfu	0 cfu

Fungal test suspension: Log 6.85.

Table 14.3: Test results showing log counts of *Trichophyton* (Ref 20MI0025171)
Date of trial: 24/11/18

Equine BIO Fluid Concentration	Contact Times					
	1 min	5 mins	15 mins	1 hour	6 hours	24 hours
1:5 Clean Conditions	Log 5.18	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:16 Clean Conditions	TNTC	TNTC	0 cfu	0 cfu	0 cfu	0 cfu
1:60 Clean Conditions	TNTC	TNTC	TNTC	0 cfu	0 cfu	0 cfu
1:5 Dirty Conditions	TNTC	0 cfu	0 cfu	0 cfu	0 cfu	0 cfu
1:16 Dirty Conditions	TNTC	TNTC	TNTC	0 cfu	0 cfu	0 cfu
1:60 Dirty Conditions	TNTC	TNTC	TNTC	Log 5.81	0 cfu	0 cfu

Fungal Test Suspension: Log 6.45. TNTC = Too Numerous To Count

In all cases, product tested was as follows: Equine BIO Fluid, batch 001, expiry 06/2022.

16.Conclusion

EBF 1:5 concentration, produced satisfactory results (> 4.0 log reduction) after 1-minute contact time under clean and dirty conditions when tested against *Streptococcus equi* (Ref 20MI010878) & *Aspergillus niger* (Ref 20EN004535). **EBF** 1:5 concentration, produced satisfactory results (> 4.0 log reduction) after 5 minutes contact time under clean and dirty conditions when tested against & *Trichophyton sp* (Ref 20MI0025171).

EBF 1:16 concentration, produced satisfactory results (> 4.0 log reduction) after 15 minutes contact time under clean and dirty conditions when tested against *Streptococcus equi*. **EBF** 1:16 concentration, produced satisfactory results (> 4.0 log reduction) after 5 minutes contact time under clean and dirty conditions when tested against *Aspergillus niger*. **EBF** 1:16 concentration, produced satisfactory results (> 4.0 log reduction) after 15 minutes contact time under clean conditions and after 1-hour contact time under dirty conditions when tested against *Trichophyton sp*.

EBF 1:60 concentration, produced satisfactory results (> 4.0 log reduction) after 1-hour contact time under clean and dirty conditions when tested against *Streptococcus equi* & *Aspergillus niger*. **EBF** 1:16 concentration, produced satisfactory results (> 4.0 log reduction) after 1-hour contact time under clean conditions and after 6 hours contact time under dirty conditions when tested against *Trichophyton sp*.

In all cases, the control test solutions (replacing the product under test with equivalent amounts of water), showed the organism counts as too numerous to count, showing the bactericidal / yeasticidal activity was due to the product under test. The controls also showed that in all cases, strains maintained heavy growths during the entire phase of testing, indicating the log reductions achieved were due to the bactericidal / yeasticidal activity of the product under test.

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Dated: 10/12/2020

APPENDIX 1

Phase 2, Step 1 - Flow Chart of Test Procedure