

資料番号 20

Bacillus subtilis DB9011株の
ラットを用いる単回経口毒性試験

DB9011のラットを用いる単回経口投与毒性試験

最終報告書

提出日 平成6年1月20日

株式会社日本バイオリサーチセンター
羽島研究所

NBR-1

試験概要 (試験番号: 4163)

表 題	DB9011のラットを用いる単回経口投与毒性試験
試験番号	4163
被験物質名	DB9011
試験系	Crj:CD (SD)系ラット (SPF)
試験委託者	
試験施設	株式会社日本バイオリサーチセンター 羽島研究所 岐阜県羽島市福寿町間島6丁目104番地
試験目的	DB9011の安全性に関する試験の一環として、平成4年3月18日付4審A第201号「飼料添加物の評価基準の制定についての別添、飼料添加物の評価基準」に基づき、DB9011を雌雄ラットに1回経口投与し、その毒性について検討した。なお、当試験は昭和63年7月29日付63審A第3039号「飼料添加物の動物試験の実施に関する基準について」およびその改正基準に従って実施した。
試験期間	平成5年10月27日～平成6年1月20日
資料保管場所	株式会社日本バイオリサーチセンター 羽島研究所 当試験により得られた資料は、最終報告書提出後5年間は株式会社日本バイオリサーチセンター 羽島研究所の資料保管室に保管する。その後は、試験委託者と協議の上決定する。
試験計画書からの逸脱	動物発注匹数は雌雄各25匹としたが、動物供給業者の都合により雌雄各28匹を入手した。しかし、この変更が試験の実施に支障を及ぼすことはないと判断された。 そのほか、試験計画書からの逸脱は認められなかった。

試験従事者、業務分担および署名・捺印（試験番号：4103）

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（試験計画書の作成、試験の統轄、データのチェック、最終報告書の作成）

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試験責任者 太田隆雄 (印) 平成6年1月20日
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要 約

DB9011の20、200および2000mg/kgをSprague-Dawley系の雌雄ラット各5例に1回経口投与し、その毒性を検討した。なお、対照として、注射用水を同様に投与した。

(1) 死亡例は雌雄いずれの群にも認められず、DB9011の最小致死量は2000mg/kg以上と判断された。

(2) 一般状態、体重推移および剖検では、雌雄いずれもDB9011の投与による影響は認められなかった。

以上のように、2000mg/kgにおいてもDB9011の投与による影響は認められず、その安全性は高いと考えられた。

緒 言

DB9011は[REDACTED]で飼料添加物として開発中の枯草菌乳糖製剤である。今回、その安全性に関する試験の一環として、平成4年3月16日付4畜A第201号「飼料添加物の評価基準の制定についての別添、飼料添加物の評価基準」に基づき、DB9011を雌雄ラットに1回経口投与し、その毒性について検討した。なお、当試験は昭和63年7月20日付63畜A第3039号「飼料添加物の動物試験の実施に関する基準について」およびその改正基準に従って実施した。

被験物質

DB9011 (ロット番号: 3001-930930-11P) は、Bacillus subtilis DB9011株の耐熱胞子を 10^{10} 個/gの割合で乳糖に添加した白色の粉末であり、平成5年10月20日[REDACTED]株式会社から40g提供された(Attachment 1)。安定性試験については、現在試験委託者で継続中であるが、加速試験により少なくとも3カ月間は菌数の低下はなく安定であることが確認されている(Attachment 1)。入手後、これらは試験施設の被験物質保管室の保管庫内に冷所(1~10℃)・遮光の条件下で保存した。

媒体として、注射用水(ロット番号: 2K94P、メーカー名: 株式会社大塚製薬工場)を用いた。投与検体は、被験物質に必要な量の注射用水を加え、濃度分布がほぼ均一となるようにマグネティックスターラーで攪拌することにより懸濁液を調製した。なお、被験物質の媒体中での安定性は確認されていないため、調製は用時に行った。投与量の算出は、原末重量で行った。

試験委託者から提示された資料に基づいて作成した測定法に準じて投与日に各投与検体中のDB9011株生菌数を測定した。その結果、投与検体1ml中の生菌数は、2mg/ml溶液が 0.8×10^7 個、20mg/ml溶液が 1.2×10^8 個、200mg/ml溶液が 1.0×10^9 個であった(Attachment 2)。

残余の投与検体は廃棄した。また、残余被験物質は投与終了後にすべて試験委託者に返却した。

試験方法

1. 試験動物および飼育条件

試験には、毒性試験に一般的に用いられている動物種で、その系統維持が明らかなSprague-Dawley系の雌雄ラット[Crj:CD(SD),SPF]を使用した。

動物は、平成5年10月27日に雌雄各26匹を、4週齢で日本チャールス・リバー株式会社日野飼育センターから購入した。動物の入手後2日の体重範囲は、雄が75~93g、雌が75~85gであった。入手したラットは5日間の検疫期間およびその後3日間の馴化期間を設け、この間4回の体重測定と一般状態の観察を毎日行い、異常がみられない5週齢の動物を試験に用いた。飼育は、室温20~24℃、湿度40~70%、明暗各12時間(照明:午前6時~午後6時)、換気回数12回/時間(フィルターにより除菌された新鮮空気)に設定された動物飼育室

5. 群構成および投与量

群構成および投与量を以下に示した。各群の動物数は、雌雄ともに5例とした。

試験群	投与量 (濃度)	動物数 (動物番号)	
		雄	雌
第1群 対照 (注射用水)	0mg/kg (0%)	5 (001~005)	5 (051~055)
第2群 DB9011	20mg/kg (0.2%)	5 (101~105)	5 (151~155)
第3群 DB9011	200mg/kg (2%)	5 (201~205)	5 (251~255)
第4群 DB9011	2000mg/kg (20%)	5 (301~305)	5 (351~355)

6. 投与量設定の理由

DB9011は枯草菌の乳糖製剤であり、その安全性は高いと考えられた。したがって、当試験の高用量は安全性試験における上限用量とされている2000mg/kgとした。また、枯草菌生菌はDB9011 1g中に 10^{10} 個含まれていることから、毒性が出現した場合に用量依存性のある変化を捉え易くする目的で公比を10とし、200および20mg/kgを中用量および低用量に設定した。

対照として、注射用水を同様に10ml/kg投与する群を別に設けた。

7. 観察および検査項目

(1) 観察期間

投与後14日間とした。

(2) 一般状態

投与日は投与前および投与後0.5、2、4および6時間に、投与翌日から最終観察日までは1日1回、一般状態を観察した。

(3) 体重測定

投与日 (投与直前) および投与後1日、3日、7日、10日および14日に測定した。

(4) 剖検

観察期間終了時に全例をエーテル麻酔下で腹大動脈から放血致死させたのちに剖検した。剖検で異常が認められた器官・組織は、10%中性緩衝ホルマリン液に固定し保存することとしたが、該当器官・組織はなかった。

8. 統計学的方法

LD₅₀値は算出せず、試験結果から最小致死量を推定した。体重は、各群で平均値および標準偏差を算出し、続いてBartlett法による等分散性の検定を行った。雌雄いずれの測定日の体重も等分散であったため一元配置法による分散分析を行った結果、有意差は認められなかった。したがって、それ以降の統計処理は行われなかった。なお、有意水準は危険率5%未満 ($p < 0.05$) とした。

試験実施日

以下に、当試験の実施日を記す。

動物入手日	平成 5年10月27日
群分け日	平成 5年11月 4日
投与日	平成 5年11月 4日
観察終了日 (剖検日)	平成 5年11月18日

なお、当試験の実施期間中に、試験の信頼性に悪影響を及ぼす疑いのある予期しえなかった事態は認められなかった。

試験成績

1. 経日死亡および致死量

経日死亡および概略の致死量をTable 1に示した。

死亡例は雌雄いずれの群にも認められず、最小致死量は2000mg/kg以上と判断された。

2. 一般状態

一般状態の観察結果を、雄はTable 2 (Appendix 1-1~1-4) に、雌はTable 3 (Appendix 2-1~2-4) に示した。

雌雄いずれの群も一般状態に異常は認められなかった。

3. 体重

体重推移を、雄はFig. 1およびTable 4 (Appendix 3-1~3-4) に、雌はFig. 2およびTable 5 (Appendix 4-1~4-4) に示した。

DB9011の各投与群は雌雄いずれも対照群とほぼ同様の推移を示し、いずれの測定日の体重にも対照群との間に有意差は認められなかった。

4. 剖検

剖検所見を、雄はTable 6 (Appendix 5-1~5-4) に、雌はTable 7 (Appendix 6-1~6-4) に示した。

雌雄いずれの群においても異常は認められなかった。

結 論

DB9011の20、200および2000mg/kgをSprague-Dawley系ラットの雌雄に1回経口投与して、その毒性を検討した。

DB9011の各投与群では、雌雄いずれも死亡発現はみられず、一般状態、体重推移および剖検でも被験物質の投与による影響は認められなかった。したがって、DB9011のラットでの最小致死量は2000mg/kg以上であり、その安全性は高いと考えられた。

1. DB9011菌原体データのデータ

① サンプル Lot NO. 3001-930930-02P

② 菌数: 1.0×10^{10} cfu/g

③ 安定性: 原体-保存安定性試験を実施中です。

加速試験 (期間6ヶ月、40℃湿度75%) では10月現在
(3ヶ月目) 菌数の低下は無く安定です。

懸濁液-懸濁液の安定性データは有りません。

乳糖が入っているため室温条件では数時間で発芽すると思われ
ますので実験の都度試料を調整する必要が有ります。

④ 保管条件: 冷暗所

2. DB9011菌の定量法 (希釈液及び培地は生菌剤試験法に準ずる)

で行っている粉体中の菌の定量法ですので、実験に合うように改変してください

準備

希釈液: カゼイン製ペプトン 1g, NaCl 5g, Tween 80 1g

水 1L (pH6.9~7.1),

原体希釈用に、デュラン瓶等に希釈液100mlと攪拌子をいれオートクレーブする

10倍希釈用に、希釈液9mlを試験管にいれオートクレーブする

寒天培地: カゼイン製ペプトン 10g, 肉エキス 5g, NaCl 5g,

寒天 15g, 水 1L (pH6.9~7.1)

これをオートクレーブ後、滅菌シャーレに約20mlずつ分注する。

操作法

① 原体希釈: 試料1gを精密に量り、これを上記の希釈液100mlに加え、スターラ
で良く混ぜ (約10分)、これを試料原液とする。

② 10倍希釈: 試料原液1mlを希釈液9mlに加えミキサー等で攪拌する。

この操作を繰り返し、1ml中に生菌が30~300個含む濃度に調整し、これを
試料溶液とする。

③ 試料溶液0.1mlずつを5枚の寒天プレートにコンラージし、38℃で24時間培
養し、出現したコロニーを数え、平均コロニー数を求める。

以上

試 験 成 績 書

— 投与検体の生菌数確認 —

試験番号：4163
 被験物質：DB9011原体
 媒 体：注射用水
 調製日：平成5年11月4日
 測定日：平成5年11月4日

測定結果

濃 度 (mg/ml)	コニ-数/プレート			試料溶液1ml 中の生菌数 (cfu/ml)
	1回目	2回目	平均	
2	57 59 82	86 90 102	80	0.8×10^7
	66	93		
20	106 109 120	107 120 125	115	1.2×10^8
	112	117		
200	95 103 107	82 91 99	97	1.0×10^9
	102	91		

測定者 永澤 佳子 (印) 平成 5 年 11 月 8 日

被験物質
管理責任者 坂野 浩平 (印) 平成 5 年 11 月 8 日

株式会社日本バイオリサーチセンター 羽島研究所

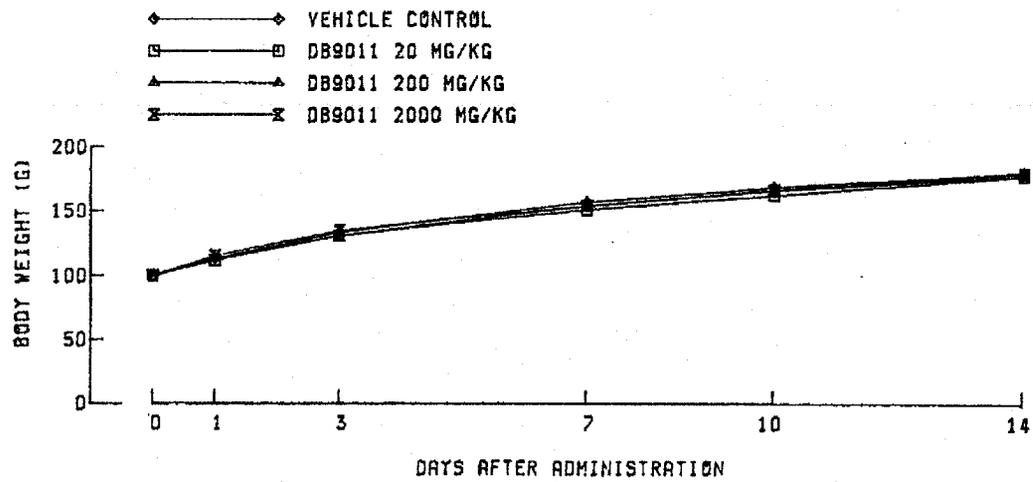


Fig. 2. Body weight of female rats in single dose toxicity study of DB9011 by oral administration.

Table 4. Body weight of male rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control		DB9011					
	0		20		200		2000	
Number of males	5		5		5		5	
Days after administration								
0	112.4 ± 2.6		113.0 ± 3.3		112.4 ± 3.0		112.8 ± 2.7	
1	128.6 ± 3.2		129.0 ± 2.9		130.0 ± 3.5		129.0 ± 4.1	
3	151.0 ± 4.9		154.4 ± 2.2		151.8 ± 4.7		152.2 ± 6.0	
7	188.4 ± 9.0		191.0 ± 3.7		188.8 ± 8.5		190.0 ± 12.1	
10	214.0 ± 12.1		218.4 ± 5.1		213.8 ± 11.1		216.6 ± 15.5	
14	246.4 ± 13.7		252.2 ± 7.7		245.4 ± 14.2		250.2 ± 21.7	

Each value shows mean (g) ± S.D.

Table 6. Necropsy finding of male rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control	DB9011		
	0	20	200	2000
Number of males	5	5	5	5
Normal	5	5	5	5

Table 7. Necropsy finding of female rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control	DB9011		
	0	20	200	2000
Number of females	5	5	5	5
Normal	5	5	5	5

Appendix : 1 - 1. Individual general sign of male rats

Test No.4163

(Group : Vehicle control)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
002	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
004	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
005	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 1 - 3. Individual general sign of male rats

Test No.4163

(Group : DB9011 200 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
201	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
202	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
203	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
204	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
205	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 1 - 4. Individual general sign of male rats

Test No.4163

(Group : DB9011 2000 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
301	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
302	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
303	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
304	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
305	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
O	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

O: Normal.

Appendix: 2 - 1. Individual general sign of female rats

Test No.4163

(Group: Vehicle control)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
051	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
052	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
053	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
054	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
055	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0: Normal.

Appendix : 2 - 2. Individual general sign of female rats

Test No.4163

(Group : DB9011 20 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
151	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
152	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
153	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
154	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
155	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
O	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

O : Normal.

Appendix : 2 - 3. Individual general sign of female rats

Test No.4163

(Group : DB9011 200 mg/kg)

Animal No.	Hours after administration				Days after administration														
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
251	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
252	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
253	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
254	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
255	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	

0 : Normal.

Appendix : 2 - 4. Individual general sign of female rats

Test No.4163

(Group : DB9011 2000 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0~0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
351	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
352	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
353	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
354	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
355	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
O	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

O : Normal.

Appendix 3-1. Individual body weight (g) of male rats

Test No. 416

(Group: Vehicle control)

Male No.	Days after administration					
	0	1	3	7	10	14
001	109	125	147	180	205	235
002	113	129	151	182	208	241
003	116	133	159	203	233	270
004	111	126	151	189	205	241
005	113	130	147	188	219	245
Number of males	5	5	5	5	5	5
Mean	112.4	128.6	151.0	188.4	214.0	246.4
S.D.	2.6	3.2	4.9	9.0	12.1	13.7

Appendix 3-2. Individual body weight (g) of male rats

Test No. 416.

(Group: DB9011 20 mg/kg)

Male No.	Days after administration					
	0	1	3	7	10	14
101	109	128	152	189	219	253
102	118	133	154	188	216	249
103	114	131	158	196	224	261
104	112	127	154	188	211	241
105	112	126	154	194	222	257
Number of males	5	5	5	5	5	5
Mean	113.0	129.0	154.4	191.0	218.4	252.2
S.D.	3.3	2.9	2.2	3.7	5.1	7.7
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 3-3. Individual body weight (g) of male rats

Test No. 416

(Group: DB9011 200 mg/kg)

Male No.	Days after administration					
	0	1	3	7	10	14
201	115	134	155	198	225	253
202	116	133	158	198	226	264
203	109	127	146	181	206	242
204	110	130	150	185	210	242
205	112	126	150	182	202	226
Number of males	5	5	5	5	5	5
Mean	112.4	130.0	151.8	188.8	213.8	245.4
S.D.	3.0	3.5	4.7	8.5	11.1	14.2
Judge Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 3-4. Individual body weight (g) of male rats

Test No. 416

(Group: DB9011 2000 mg/kg)

Male No.	Days after administration					
	0	1	3	7	10	14
301	113	124	151	188	213	246
302	117	133	158	201	233	273
303	110	126	143	170	192	216
304	111	129	152	195	223	253
305	113	133	157	196	222	263
Number of males	5	5	5	5	5	5
Mean	112.8	129.0	152.2	190.0	216.6	250.2
S. D.	2.7	4.1	6.0	12.1	15.5	21.7
Judge Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 4-1. Individual body weight (g) of female rats

Test No. 416.

(Group: Vehicle control)

Female No.	Days after administration					
	0	1	3	7	10	14
051	99	110	130	150	164	174
052	99	113	131	151	161	179
053	103	118	138	169	183	199
054	98	111	130	148	160	174
055	100	115	140	168	179	182
Number of females	5	5	5	5	5	5
Mean	99.8	113.4	133.8	157.2	169.4	181.6
S.D.	1.9	3.2	4.8	10.4	10.8	10.3

Appendix 4-2. Individual body weight (g) of female rats

Test No. 414

(Group: DB9011 20 mg/kg)

Female No.	Days after administration					
	0	1	3	7	10	14
151	100	110	130	150	158	172
152	103	117	135	151	160	176
153	99	113	132	151	162	178
154	96	107	127	149	161	177
155	103	113	132	155	174	190
Number of females	5	5	5	5	5	5
Mean	100.2	112.0	131.2	151.2	163.0	178.6
S. D.	2.9	3.7	2.9	2.3	6.3	6.8
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 4-3. Individual body weight (g) of female rats

Test No. 416

(Group: DB9011 200 mg/kg)

Female No.	Days after administration					
	0	1	3	7	10	14
251	99	113	130	150	163	172
252	103	114	132	155	166	178
253	99	113	132	159	174	187
254	96	111	122	148	161	175
255	102	115	140	159	171	182
Number of females	5	5	5	5	5	5
Mean	99.8	113.2	131.2	154.2	167.0	178.8
S.D.	2.8	1.5	6.4	5.1	5.4	5.9
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 4-4. Individual body weight (g) of female rats

Test No. 416

(Group: DB9011 2000 mg/kg)

Female No.	Days after administration					
	0	1	3	7	10	14
351	99	115	132	153	162	176
352	99	115	134	150	168	179
353	103	121	142	162	171	189
354	101	113	134	150	159	170
355	98	113	134	156	176	191
Number of females	5	5	5	5	5	5
Mean	100.0	115.4	135.2	154.2	167.2	181.0
S.D.	2.0	3.3	3.9	5.0	6.8	8.9
Judge Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 5-1. Individual necropsy finding of male rats

Test No. 4163

(Group: Vehicle control)

Male No.	Necropsy finding
001	Normal
002	Normal
003	Normal
004	Normal
005	Normal

Appendix 5-2. Individual necropsy finding of male rats

Test No. 4163

(Group: DB9011 20 mg/kg)

Male No.	Necropsy finding
101	Normal
102	Normal
103	Normal
104	Normal
105	Normal

Appendix 5-3. Individual necropsy finding of male rats

Test No. 4163

(Group: DB9011 200 mg/kg)

Male No.	Necropsy finding
201	Normal
202	Normal
203	Normal
204	Normal
205	Normal

Appendix 5-4. Individual necropsy finding of male rats

Test No. 4163

(Group: DB9011 2000 mg/kg)

Male No.	Necropsy finding
301	Normal
302	Normal
303	Normal
304	Normal
305	Normal

Appendix 6-1. Individual necropsy finding of female rats

Test No. 4163

(Group: Vehicle control)

Female No.	Necropsy finding
051	Normal
052	Normal
053	Normal
054	Normal
055	Normal

Appendix 6-2. Individual necropsy finding of female rats

Test No. 4163

(Group: DB9011 20 mg/kg)

Female No.	Necropsy finding
151	Normal
152	Normal
153	Normal
154	Normal
155	Normal

Appendix 6-3. Individual necropsy finding of female rats

Test No. 4163

(Group: DB9011 200 mg/kg)

Female No.	Necropsy finding
251	Normal
252	Normal
253	Normal
254	Normal
255	Normal

Appendix 6-4. Individual necropsy finding of female rats

Test No. 4163

(Group: DB9011 2000 mg/kg)

Female No.	Necropsy finding
351	Normal
352	Normal
353	Normal
354	Normal
355	Normal

Attachment I / Translation

Material No. 20

Single Dose Oral Toxicity Study of *Bacillus subtilis* DB9011 in Rats

Single Dose Oral Toxicity Study of *Bacillus subtilis* DB9011 in Rats

Final Report

Submission: January 20, 1994

Hashima Laboratory, Nihon Bioresearch Inc.

NBR-1

Summary of study (study no.: 4163)

Study title	Toxicity study of DB9011 After Single-dose Oral Administration in Rats
Study No.	4163
Test substance	DB9011
Test animal	Crj: CD (SD) rats (SPF)
Sponsor	[REDACTED]
Testing facility	Hashima Laboratory, Nihon Bioresearch Inc. 104 Majima 6-chome, Fukuju-cho, Hashima, Gifu
Study objectives	As part of safety studies, DB9011 was orally administered once to male and female rats to investigate toxicity, in accordance with Notification No. 4-Chiku-A-201 (dated March 16, 1992) "Appendix of establishment of evaluation standards for feed additives: Evaluation standards for feed additives". The present study complied with Notification No. 63-Chiku-A-3039, dated July 29, 1988, "Good Laboratory Practice for Feed Additives" and its amended standards.
Study period	October 27, 1993 to January 20, 1994
Location of archives	Hashima Laboratory, Nihon Bioresearch Inc. Materials obtained from the present study are to be archived in the Archives of Hashima Laboratory, Nihon Bioresearch Inc. for 5 years after submission of final report. Archiving beyond the period shall be decided between Nihon Bioresearch Inc. and the sponsor.
Deviations from study protocol	Male and female rats (25 each) were to be ordered; however, for the convenience of the breeder 26 of each sex were purchased. This change was considered to have no effect on performance of the study. There were no other deviations from the protocol.

Personnel engaged in the study, work assignment, and signatures (study No. 4163)

Study director: Takao Ota

(Preparation of study protocol, supervision of study, data check, preparation of final report)

Study leader: Takashi Fujimura

(Direction of study practice, observation of general sign, general control of animals, data check)

Staff members: Kazuyoshi Naito, Mikihiro Nishiwaki, Emi Yamaguchi, Yoshiko Kato, Hiromi Tsuda

(Administration of samples, observation of general sign, general control of animals)

Tsuneo Koike, Emi Tsuda, Naomi Yamauchi

(Necropsy)

Kohei Makino, Kiyoka Oku, Yoshiko Nagasawa*

(Storage and control of test substance, preparation of dosing samples, *measurement of live bacteria in dosing sample)

Akiyoshi Yamamoto

(Statistical analysis)

Study Director (preparation of final report)

Signature: _____

Takao Ota

Date: January 20, 1994

Administrator

Signature: _____

Hisao Iwata

Date: January 20, 1994

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Abstract

DB9011 was orally administered once to Sprague-Dawley rats (5 each of both sexes) at 20, 200, and 2000 mg/kg to investigate toxicity. Water for injection was administered as a control substance in the same manner.

- (1) No animal deaths occurred in any group for both sexes, therefore minimum lethal dose of DB9011 was estimated to be greater than 2000 mg/kg.
- (2) DB9011 had no effect on general sign, time-course changes in body weight, and necropsy for both sexes.

Administration of DB9011 had no effect up to 2000 mg/kg, therefore its safety was considered to be excellent.

Introduction

DB9011 is a *Bacillus subtilis* lactose formulation under development as a feed additive by [REDACTED]. As part of safety studies, DB9011 was orally administered once to male and female rats to investigate toxicity, in accordance with Notification No. 4-Chiku-A-201 (dated March 16, 1992) "Appendix of establishment of evaluation standards for feed additives: Evaluation standards for feed additives". The present study complied with Notification No. 63-Chiku-A-3039, dated July 29, 1988, "Good Laboratory Practice for Feed Additives" and its amended standards.

Test Substance

DB9011 (lot No.: 3001-930930-11P) is a white powder mixture of lactose and heat-stable spores of *Bacillus subtilis* DB9011 at a ratio of 10^{10} spores/g. The test substance (40 g) was provided [REDACTED] on October 20, 1993 (Attachment 1). A stability study is currently being performed by the sponsor; however, an accelerated study has confirmed that DB9011 is stable for at least 3 months without a decrease in the number of bacteria (Attachment 1). After receipt, the test strain was stored in a cold place (1 to 10°C, test substance storage room of the testing facility), protected from light.

Water for injection (lot No.: 2K94P; Otsuka Pharmaceutical Factory, Inc.) was used as a vehicle control. Test strain was suspended in a specified volume of water for injection, then stirred with a magnetic stirrer for homogenous dispersion and used as dosing suspension. As stability of the test substance in vehicle has not been established, dosing suspensions were prepared immediately before use. Doses were calculated in terms of bulk powder weight.

In accordance with the method for measurement established based on materials provided by the sponsor, live DB9011 bacteria in each dosing sample were counted on the day of administration. Number of live bacteria in 1 mL of dosing suspension were 0.8×10^7 , 1.2×10^8 , and 1.0×10^9 in 2, 20, and 200 mg/mL dosing suspensions, respectively (Attachment 2).

Unused portions of dosing samples were discarded. Remaining test strain was returned to the sponsor after completion of administration.

Study Methods

1. Animals used for study and rearing environment
Sprague-Dawley [Crj:CD(SD), (SPF)] male and female rats were used, as they are commonly used in toxicity studies and the methods for maintenance of the strain have been established. Animals (26 each from both sexes) were purchased from Hino Breeding Center, Charles River Japan, Inc. at 4 weeks of age on October 27, 1993. Body weight ranged from 75 to 93 g and 75

to 85 g for males and females, respectively, during the 2-day period after receipt. Body weight was measured 4 times and general sign was observed everyday during a 5-day quarantine period and a following 3-day acclimatization period. Animals with no abnormal findings were used for the study at the age of 5 weeks. Animals were reared in an animal room (room 10, wing E) maintained at 20 to 24°C and 40 to 70% humidity, with a 12-hr light-dark cycle (lights on: 6:00 to 18:00), ventilated 12 cycles per hour with fresh air (sterilized by filtration through a filter). Animals were housed together (a maximum of 5 animals per cage) in stainless steel suspension type cages (240^W × 380^D × 200^H mm) during the quarantine/acclimatization period. After group assignment, animals were individually housed in stainless steel cages with 5 individual partitions (755^W × 210^D × 170^H mm). Waste elimination trays for cages and drinking bottles were replaced at least twice weekly, and stainless steel suspension type cages and stainless steel partition cages (including feeders) were replaced at least once in two weeks. Solid feed (CRF-1, Oriental Yeast Co., Ltd.) purchased within the previous 3 months was provided *ad libitum* via a feeder, except for an approximately 19-hr period from the evening on the day prior to administration and an approximately 6 hr period after administration, during which animals were fasted. Tap water was provided *ad libitum* via drinking bottles, although water supply was cut off for approximately 6 hr after administration. Analytical results for feed use in the study were obtained from Japan Food Research Laboratories and Oriental Yeast Co., Ltd. Results of water quality examination (performed approximately every 3 months) were obtained from Gifu Research Center for Public Health. Both feed and drinking water had values within the standards established by the testing facility.

2. Group assignment

Animals were stratified based on body weight using a computer on the day of administration, then assigned to each group using a random sampling method so that mean values and variance of body weight was balanced between groups. Remaining animals after group assignment were euthanized under ether anesthesia on the day of administration.

3. Individual identification

Animals were marked with a waterproof pen and their fur was dyed with pigment on the receipt date for individual identification during the quarantine/acclimatization period. After group assignment, fur dyeing with pigment and ear punching were concurrently used for identification. Labels with a description of study No., receipt date, sex and animal acclimatization No. were hung on each cage during the quarantine/acclimatization period. After group assignment, color-coded labels with description of study No., dose, and animal No. were used.

4. Administration

(1) Administration route

As DB9011 is proposed for use as a food additive, oral administration was selected.

(2) Administration method

Following the method commonly used in the testing facility, oral gavage administration was conducted using a plastic disposable syringe with an orogastric metal tube for rats.

(3) Dosing volume, plus frequency and timing of administration

Individual dosing volume was calculated based on body weight measured immediately before administration (10 mL/kg). Administration was conducted once between 10:00 a.m. and 11:00 a.m.

Body weight ranged from 109 to 118 g and 96 to 103 g, for males and females, respectively, immediately before administration.

5. Group composition and doses

Group composition and doses are shown below. Each group comprised 5 each of both sexes.

Study groups	Doses (concentrations)	No. of animals (animal number)	
		Males	Females
Group 1 control (water for injection)	0 mg/kg (0%)	5 (001 – 005)	5 (051 – 055)
Group 2 DB9011	20 mg/kg (0.2%)	5 (101 – 105)	5 (151 – 155)
Group 3 DB9011	200 mg/kg (2%)	5 (201 – 205)	5 (251 – 255)
Group 4 DB9011	2000 mg/kg (20%)	5 (301 – 305)	5 (351 – 355)

6. Rationale for selection of doses

As DB9011 is a *Bacillus subtilis* lactose formulation, its safety was presumed to be excellent. Therefore, in the present study the highest dose was set at 2000 mg/kg, specified as the upper limit for safety studies. As DB9011 contains 10^{10} live *Bacillus subtilis* per gram, 200 and 20 mg/kg were established as the middle and lowest doses by dividing 2000 by a common factor of 10, so that dose-dependency of toxicities could be easily traced.

A group receiving 10 mL/kg of water for injection was established as the control.

7. Observation and examination parameters

(1) Observation period

For 14 days after administration

(2) General sign

General sign was observed before and 0.5, 2, 4 and 6 hr after administration on the day of administration, and once daily thereafter, from the day following administration until the final observation day.

(3) Measurement of body weight

Body weight was measured on the day of administration (immediately before administration), plus 1, 3, 7, 10 and 14 days after administration.

(4) Necropsy

All animals were sacrificed by exsanguination via the abdominal aorta under ether anesthesia at completion of the observation period, then necropsied. If organs and tissues had abnormal findings at necropsy, they were to be fixed in 10% neutral buffered formalin solution and stored; however, there were no abnormalities observed.

8. Statistical analysis

LD₅₀ values were not calculated, although minimum lethal dose was estimated from the study results. Mean and standard deviation of body weight were calculated for each group, then homoscedasticity was tested by Bartlett's test. As body weight on each of the measurement days was homoscedastic for both sexes, one-way analysis of variance was conducted, however no significant differences were found. Therefore, no further statistical analysis was conducted. Significance level was set at less than 5% ($p < 0.05$).

Experimental Days

Study procedures were performed on the days recorded below.

Animal receipt:	October 27, 1993
Group assignment:	November 4, 1993
Administration:	November 4, 1993
Completion of observation (necropsy):	November 18, 1993

There were no unforeseeable events that might adversely affect reliability of the study during the study period.

Study Results

1. Animal deaths and lethal dose

Table 1 indicates the number of animal deaths over time and estimated lethal dose.

No animal deaths occurred in any group for both sexes, therefore minimum lethal dose was estimated to be at least 2000 mg/kg.

2. General sign

Table 2 (Appendices 1-1 to 1-4) and Table 3 (Appendices 2-1 to 2-4) indicate observation results for general sign of males and females, respectively.

There were no abnormal findings in any group for both sexes.

3. Body weight

Fig. 1 and Table 4 (Appendices 3-1 to 3-4) and Fig. 2 and Table 5 (Appendices 4-1 to 4-4) indicate time-course changes in body weight of males and females, respectively.

Both sexes in all DB9011 groups showed similar changes to those in the control group, with no significant difference compared to the control group on any of the measurement days.

4. Necropsy

Table 6 (Appendices 5-1 to 5-4) and Table 7 (Appendices 6-1 to 6-4) list necropsy findings for males and females, respectively.

There were no abnormal findings in any group for both sexes.

Conclusion

DB9011 was orally administered once to male and female Sprague-Dawley rats at 20, 200, and 2000 mg/kg to investigate toxicity.

No animal deaths occurred in any DB9011 group for both sexes, with no effect of the test substance on general sign, time-course changes in body weight, and necropsy findings. Based on these results, minimum lethal dose of DB9011 was estimated to be at least 2000 mg/kg in rats and safety was considered to be excellent.

Attachment-1.

1. Data on native strain of DB9011

- 1) Sample: Lot No.: 3001-930930-02P
- 2) Number of bacteria: 1.0×10^{10} cfu/g
- 3) Stability

Native bacteria: A storage stability study of native bacteria is ongoing.
In an accelerated study (6 months, 40°C, humidity 75%) there was no decrease in the number of bacteria, indicating DB9011 was stable, as of October, 1993 (3rd month of the study).

Suspension: No stability data on DB9011 suspensions are available.
As DB9011 contains lactose, spores will germinate within a few hours at room temperature. Therefore, samples must be prepared immediately before experiments.

- 4) Storage conditions: Cool, dark place

2. Quantification method for strain DB9011 (dilution and culture media complied with specifications described in the study method for probiotics)

As this is the quantification method for the powder form of strain DB9011 conducted by [REDACTED], the method may be modified as appropriate for the study.

Preparation

Dilution media: Casein peptone 1 g, NaCl 5 g, Tween 80 1 g, water 1 L (pH 6.9 to 7.1)
Dilution media (100 mL) is placed into a Duran bottle containing a magnetic stirrer, then autoclaved to prepare dilution media for the bulk product.
Dilution media (9 mL) is dispensed into a tube, then autoclaved to prepare dilution media for 10-fold dilution.

Agar culture medium: Casein peptone 10 g, broth 5 g, NaCl 5 g, agar 15 g, water 1 L (pH 6.9 to 7.1)
These ingredients are autoclaved, then approximately 20 mL aliquotted into sterilized Petri dishes.

Procedures

- 1) Dilution of bulk product: Sample (1 g) is accurately weighed, then 100 mL of dilution media added and mixed using a magnetic stirrer for approximately 10 min. This suspension is used as sample stock solution.
- 2) 10-fold dilution: To 1 mL of sample stock solution, 9 mL of dilution media is added and

Attachment- 1.

mixed using a mixer.

This procedure is repeated until live bacteria concentration reaches 30 to 300 cfu per mL, then this solution is used as sample solution.

- 3) After 0.1 mL each of sample solution is evenly plated onto 5 agar plates, samples are incubated at 38°C for 24 hr, then number of colonies counted to calculate average number.

Study Results

Confirmation of number of live bacteria in dosing samples

Study No.: 4163
 Test substance: DB9011 bulk product
 Medium: Water for injection
 Preparation: November 4, 1993
 Measurement: November 4, 1993

Results of measurement

Concentration (mg/mL)	No. of colonies/plate			No. of live bacteria per mL of sample solution (cfu/mL)
	1 st	2 nd	Average	
2	57	86	80	0.8×10^7
	59	90		
	82	102		
	66	93		
20	106	107	115	0.2×10^8
	109	120		
	120	125		
	112	117		
200	95	82	97	1.0×10^9
	103	91		
	107	99		
	102	91		

Person that performed measurement: Yoshiko Nagasawa

Date: November 8, 1993

Person responsible for control of test substance: Kohei Makino

Date: November 8, 1993

Hashima Laboratory, Nihon Bioresearch Inc.

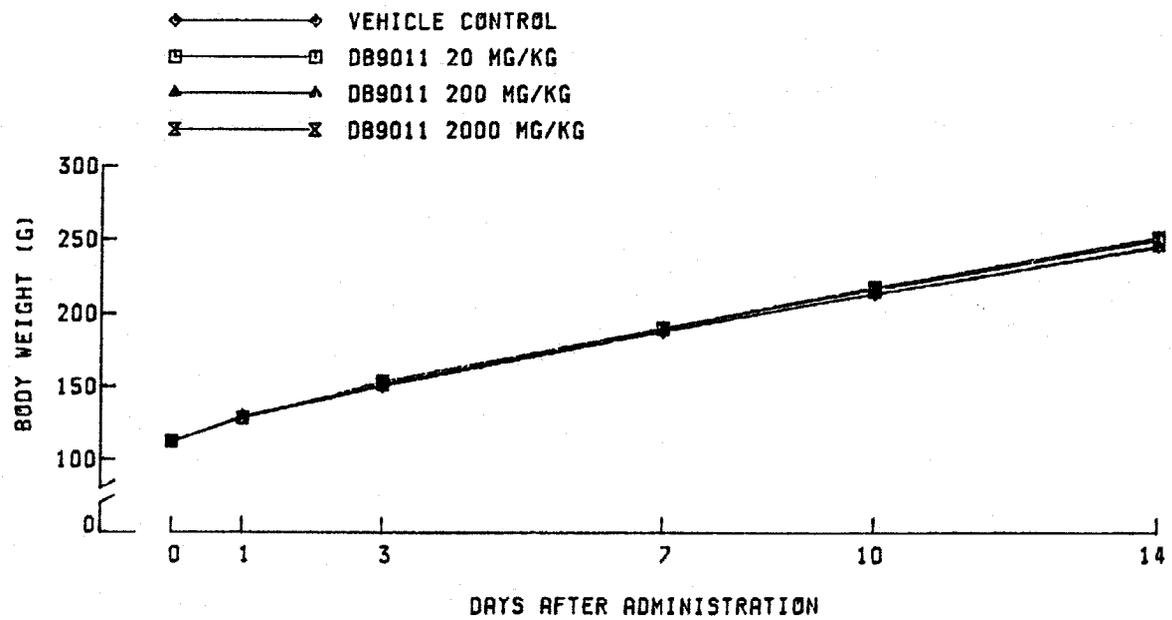


Fig. 1. Body weight of male rats in single dose toxicity study of DB9011 by oral administration.

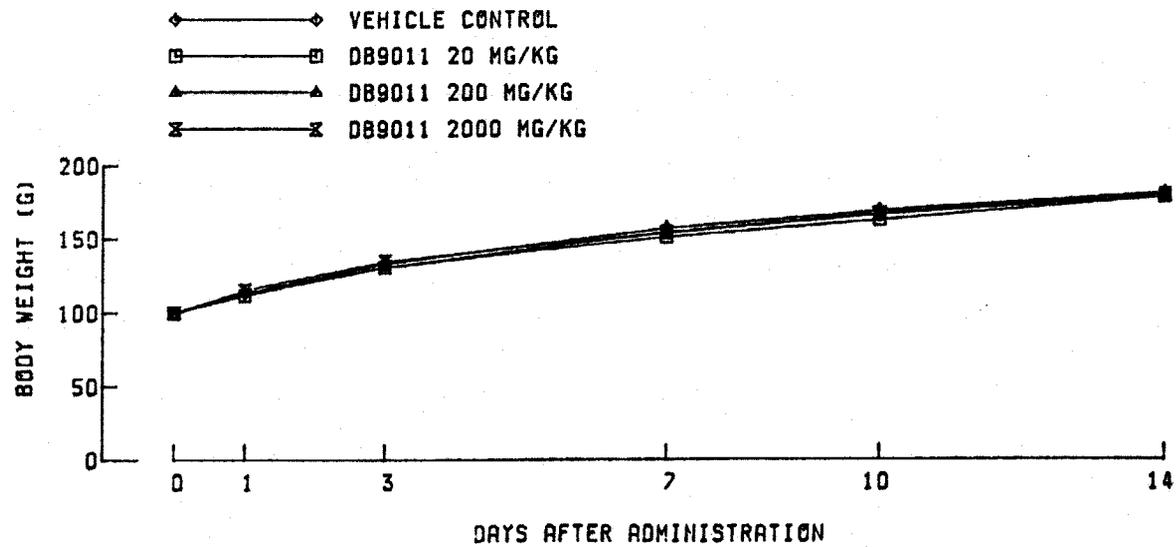


Fig. 2. Body weight of female rats in single dose toxicity study of DB9011 by oral administration.

Table 4. Body weight of male rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control		DB9011			
	0		20	200		2000
Number of males	5		5	5		5
Days after admin- istration						
0	112.4 ± 2.6		113.0 ± 3.3	112.4 ± 3.0		112.8 ± 2.7
1	128.6 ± 3.2		129.0 ± 2.9	130.0 ± 3.5		129.0 ± 4.1
3	151.0 ± 4.9		154.4 ± 2.2	151.8 ± 4.7		152.2 ± 6.0
7	188.4 ± 9.0		191.0 ± 3.7	188.8 ± 8.5		190.0 ± 12.1
10	214.0 ± 12.1		218.4 ± 5.1	213.8 ± 11.1		216.6 ± 15.5
14	246.4 ± 13.7		252.2 ± 7.7	245.4 ± 14.2		250.2 ± 21.7

Each value shows mean (g) ± S.D.

Table 5. Body weight of female rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control		DB9011					
	0		20		200		2000	
Number of females	5		5		5		5	
Days after administration								
0	99.8 ± 1.9		100.2 ± 2.9		99.8 ± 2.8		100.0 ± 2.0	
1	113.4 ± 3.2		112.0 ± 3.7		113.2 ± 1.5		115.4 ± 3.3	
3	133.8 ± 4.8		131.2 ± 2.9		131.2 ± 6.4		135.2 ± 3.9	
7	157.2 ± 10.4		151.2 ± 2.3		154.2 ± 5.1		154.2 ± 5.0	
10	169.4 ± 10.8		163.0 ± 6.3		167.0 ± 5.4		167.2 ± 6.8	
14	181.6 ± 10.3		178.6 ± 6.8		178.8 ± 5.9		181.0 ± 8.9	

Each value shows mean (g) ± S.D.

Table 6. Necropsy finding of male rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control	DB9011		
	0	20	200	2000
Number of males	5	5	5	5
Normal	5	5	5	5

Table 7. Necropsy finding of female rats in single dose toxicity study of DB9011 by oral administration

Group (mg/kg)	Vehicle control	DB9011		
	0	20	200	2000
Number of females	5	5	5	5
Normal	5	5	5	5

Appendix : 1 - 1. Individual general sign of male rats

Test No.4163

(Group : Vehicle control)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
002	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
004	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
005	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 1 - 2. Individual general sign of male rats

Test No.4163

(Group : DB9011 20 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
101	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
102	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
103	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
104	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix: 1-3. Individual general sign of male rats

Test No.4163

(Group: DB9011 200 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
201	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
202	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
203	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
204	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
205	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0: Normal.

Appendix : 1 - 4. Individual general sign of male rats

Test No.4163

(Group : DB9011 2000 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
301	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
302	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
303	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
304	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
305	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of males	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 2 - 1. Individual general sign of female rats

Test No.4165

(Group : Vehicle control)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
051	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
052	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
053	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
054	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
055	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 2 - 2. Individual general sign of female rats

Test No.4163

(Group : DB9011 20 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
151	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
152	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
153	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
154	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
155	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 2 - 3. Individual general sign of female rats

Test No.4163

(Group : DB9011 200 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
251	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
252	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
253	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
254	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
255	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix : 2 - 4. Individual general sign of female rats

Test No.4163

(Group : DB9011 2000 mg/kg)

Animal No.	Hours after administration				Days after administration													
	0-0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14
351	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
352	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
353	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
354	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
355	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of females	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

0 : Normal.

Appendix 3-1. Individual body weight (g) of male rats

Test No. 4163

(Group: Vehicle control)

Male No.	Days after administration					
	0	1	3	7	10	14
001	109	125	147	180	205	235
002	113	129	151	182	208	241
003	116	133	159	203	233	270
004	111	126	151	189	205	241
005	113	130	147	188	219	245
Number of males	5	5	5	5	5	5
Mean	112.4	128.6	151.0	188.4	214.0	246.4
S.D.	2.6	3.2	4.9	9.0	12.1	13.7

Appendix 3-2. Individual body weight (g) of male rats

Test No. 4163

(Group: 089011 20 mg/kg)

Male No.	Days after administration					
	0	1	3	7	10	14
101	109	128	152	189	219	253
102	118	133	154	188	216	249
103	114	131	158	196	224	261
104	112	127	154	188	211	241
105	112	126	154	194	222	257
Number of males	5	5	5	5	5	5
Mean	113.0	129.0	154.4	191.0	218.4	252.2
S.D.	3.3	2.9	2.2	3.7	5.1	7.7
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 3-3. Individual body weight (g) of male rats

Test No. 4163

(Group: DB9011 200 mg/kg)

Male No.	Days after administration					
	0	1	3	7	10	14
201	115	134	155	198	225	253
202	116	133	158	198	226	264
203	109	127	146	181	206	242
204	110	130	150	185	210	242
205	112	126	150	182	202	226
Number of males	5	5	5	5	5	5
Mean	112.4	130.0	151.8	188.8	213.8	245.4
S. D.	3.0	3.5	4.7	8.5	11.1	14.2
Judge Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 3-4. Individual body weight (g) of male rats

Test No. 4163

(Group: DB9011 2000 mg/kg)

Male No.	Days after administration					
	0	1	3	7	10	14
301	113	124	151	188	213	246
302	117	133	158	201	233	273
303	110	126	143	170	192	216
304	111	129	152	195	223	253
305	113	133	157	196	222	263
Number of males	5	5	5	5	5	5
Mean	112.8	129.0	152.2	190.0	216.6	250.2
S.D.	2.7	4.1	6.0	12.1	15.5	21.7
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 4-1. Individual body weight (g) of female rats

Test No. 4163

(Group: Vehicle control)

Female No.	Days after administration					
	0	1	3	7	10	14
051	99	110	130	150	164	174
052	99	113	131	151	161	179
053	103	118	138	169	183	199
054	98	111	130	148	160	174
055	100	115	140	168	179	182
Number of females	5	5	5	5	5	5
Mean	99.8	113.4	133.8	157.2	169.4	181.6
S.D.	1.9	3.2	4.8	10.4	10.8	10.3

Appendix 4-2. Individual body weight (g) of female rats

Test No. 4163

(Group: DB9011 20 mg/kg)

Female No.	Days after administration					
	0	1	3	7	10	14
151	100	110	130	150	158	172
152	103	117	135	151	160	176
153	99	113	132	151	162	178
154	96	107	127	149	161	177
155	103	113	132	155	174	190
Number of females	5	5	5	5	5	5
Mean	100.2	112.0	131.2	151.2	163.0	178.6
S.D.	2.9	3.7	2.9	2.3	6.3	6.8
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 4-3. Individual body weight (g) of female rats

Test No. 4163

(Group: DB9011 200 mg/kg)

Female No.	Days after administration					
	0	1	3	7	10	14
251	99	113	130	150	163	172
252	103	114	132	155	166	178
253	99	113	132	159	174	187
254	96	111	122	148	161	175
255	102	115	140	159	171	182
Number of females	5	5	5	5	5	5
Mean	99.8	113.2	131.2	154.2	167.0	178.8
S.D.	2.8	1.5	6.4	5.1	5.4	5.9
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 4-4. Individual body weight (g) of female rats

Test No. 4163

(Group: DB9011 2000 mg/kg)

Female No.	Days after administration					
	0	1	3	7	10	14
351	99	115	132	153	162	176
352	99	115	134	150	168	179
353	103	121	142	162	171	189
354	101	113	134	150	159	170
355	98	113	134	156	176	191
Number of females	5	5	5	5	5	5
Mean	100.0	115.4	135.2	154.2	167.2	181.0
S. D.	2.0	3.3	3.9	5.0	6.8	8.9
Judge						
Method	AN	AN	AN	AN	AN	AN

AN: Analysis of variance (one-way layout).

Appendix 5-1. Individual necropsy finding of male rats

Test No. 4163

(Group: Vehicle control)

Male No.	Necropsy finding
001	Normal
002	Normal
003	Normal
004	Normal
005	Normal

Appendix 5-2. Individual necropsy finding of male rats

Test No. 4163

(Group: DB9011 20 mg/kg)

Male No.	Necropsy finding
101	Normal
102	Normal
103	Normal
104	Normal
105	Normal

Appendix 5-3. Individual necropsy finding of male rats

Test No. 4163

(Group: DB9011 200 mg/kg)

Male No.	Necropsy finding
201	Normal
202	Normal
203	Normal
204	Normal
205	Normal

Appendix 5-4. Individual necropsy finding of male rats

Test No. 4163

(Group: DB9011 2000 mg/kg)

Male No.	Necropsy finding
301	Normal
302	Normal
303	Normal
304	Normal
305	Normal

Appendix 6-1. Individual necropsy finding of female rats

Test No. 4163

(Group: Vehicle control)

Female No.	Necropsy finding
051	Normal
052	Normal
053	Normal
054	Normal
055	Normal

Appendix 6-2. Individual necropsy finding of female rats

Test No. 4163

(Group: DB9011 20 mg/kg)

Female No.	Necropsy finding
151	Normal
152	Normal
153	Normal
154	Normal
155	Normal

Appendix 6-3. Individual necropsy finding of female rats

Test No. 4163

(Group: DB9011 200 mg/kg)

Female No.	Necropsy finding
251	Normal
252	Normal
253	Normal
254	Normal
255	Normal

Appendix 6-4. Individual necropsy finding of female rats

Test No. 4163

(Group: DB9011 2000 mg/kg)

Female No.	Necropsy finding
351	Normal
352	Normal
353	Normal
354	Normal
355	Normal

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翻訳証明書
Certificate of Translation

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This is to certify that the following document has been translated faithfully and accurately to the best of our knowledge and ability.

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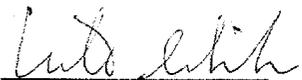
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